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## Clinical and Experimental

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# MORPHOLOGIC CHANGES IN THE LYMPHOCYTES OF PERSONS EXPOSED TO IONIZING RADIATION

ANNAMAE DICKIE AND LOUIS H. HEMPELMANN, M.D.
LOS ALAMOS, N. M.

EXAMINATION of supravital preparations of the blood of persons exposed to ionizing radiations and toxic chemicals at the Los Alamos Scientific Laboratory has revealed the presence of an unusually large number of refractive neutral red bodies in the cytoplasm of the circulating lymphocytes. These bodies are also present in smaller numbers in lymphocytes of unexposed persons. They can be distinguished from intracellular vacuoles which also stain with neutral red. The increase in neutral red bodies is evident even when exposure to radiation is within the tolerance range, eq., not greater than 0.1 roentgen of x-rays or gamma rays per day delivered to the entire body. The neutral red bodies are found in increased numbers after acute exposure to doses of ionizing radiation which do not affect the total white blood cell count, the total lymphocyte count, or the differential blood cell picture.

## METHODS AND MATERIALS

This study covers a two-year period from 1944 to 1946. All of the subjects have been employed at the Los Alamos Scientific Laboratory. Except for those in control group, all persons have been engaged in work which has resulted in exposure to ionizing radiation or toxic chemicals. With rare exceptions, the exposure was limited to one type of ionizing radiation or to one kind of chemical. The subjects included in this report were carefully selected for uniformity of sex and age. The conditions under which the study was carried out were kept as uniform as possible. The subjects were healthy, adult men, the great majority of whom were between 20 and 30 years of age. All were in residence at the Los Alamos project. All subjects were essentially of the same economic status. Each blood sample was taken in the morning between eight-thirty and ten o'clock. Excluded from this series was any subject showing evidence of illness (except for a group of ambulatory controls specifically chosen for upper respiratory infections), extreme fatigue, or hematologic abnormalities of known or unknown etiology (except radiation).

As far as can be ascertained, dissimilarities between the control and the exposed subjects were limited to occupation and number of blood counts per subject. All exposed persons were engaged in laboratory work of some sort,

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while the occupations of the control group ranged from physical work performed out-of-doors to clerical work. Relatively few persons in the control series worked under conditions comparable to those under which the exposed subjects worked. As far as the number of blood counts per subject is concerned, not more than one or, at the most, two blood counts were obtained for each control, while monthly or bimonthly blood counts were done on the exposed personnel. In many cases this was the only blood count which a control subject had experienced. The emotional response in some of these individuals cannot be ruled out as a factor which may have modified their blood pictures.

Every practical means was used to minimize technical errors and to reduce the statistical variation of individual counts. All pipettes and hemocytometer chambers used in this study were standardized by the United States Bureau of Standards. The red blood cell count was determined by examining two sides of a hemocytometer chamber filled with well-mixed diluted blood from a single pipette. Both sides of two chambers containing mixed diluted blood from two pipettes were counted to determine the white blood cell count. The hemoglobin content of the blood was estimated by means of the oxyhemoglobin method of Evelyn.[1] The differential leucocyte picture was obtained by examining 300 cells—200 on a dried film of blood stained with Wright's stain, and 100 in supravital preparations. In most cases, the number of refractive neutral red bodies in fifty lymphocytes was counted and recorded.

Since the morphologic change in the lymphocytes reported in this article can be visualized only in supravital preparations, a detailed account of this technique as used in the Los Alamos Medical Laboratory will be given:

Microscopic slides and cover slips are carefully cleaned with potassium dichromate-sulfuric acid solution. They are then rinsed with distilled water, soaked several days in ethyl alcohol, dried, and stored in a dust-free box. A small drop of blood from a fresh stab incision on the finger or lobe of the ear is picked up with a clean cover slip (No. 0). The glass is inverted and allowed to fall on a microscopic slide on which an alcoholic solution of neutral red and Janus green has dried. When the blood has spread between the two surfaces to form a thin film, the cover slip is rimmed with petroleum jelly. The preparation is kept at 10 to 15° C. until just before examination, when it is allowed to come to room temperature.

In order to obtain adequate uptake of the dyes by the leucocytes, it has been necessary to use much stronger solutions of stain than those recommended in the literature.[2] The dye solution used in this laboratory consists of 4.0 ml. of a saturated alcoholic solution of Janus green and 16.4 ml. of a saturated alcoholic solution of neutral red dissolved in 100 ml. of absolute ethyl alcohol freshly prepared by the sodium phthalate method.[3] The alcoholic solution is allowed to flow onto clean microscopic slides and the excess is wiped off immediately. The slides are dried and stored in light-proof, dust-free containers. Freshly stained slides are prepared every forty-eight hours. Presumably, the intensity of scattered sunlight at this altitude (7,300 feet above sea level) modifies the dyes in an unknown manner which makes necessary the use of higher concentrations of staining solutions. The appearance of the cells and

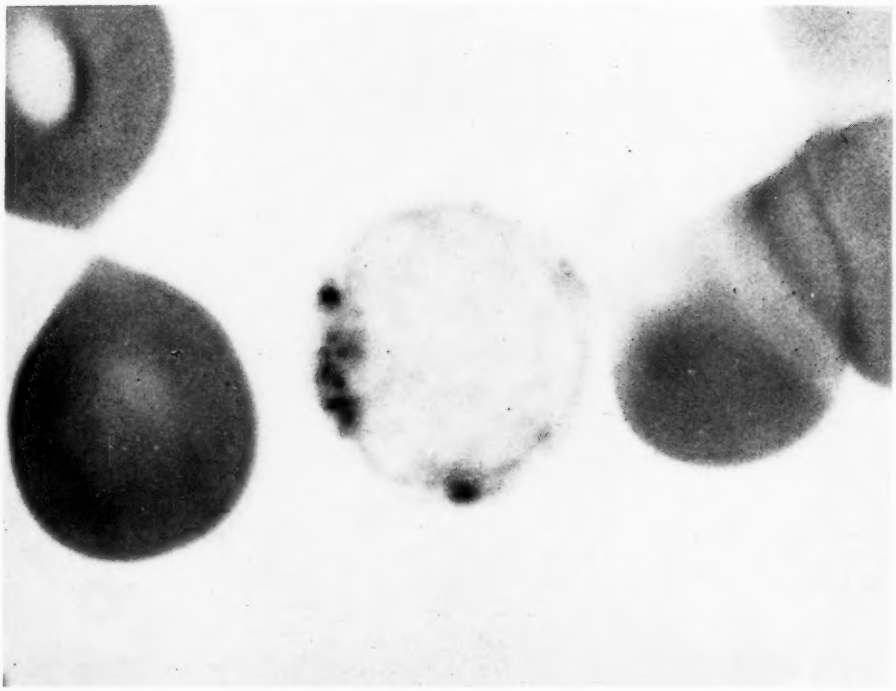

Fig. 1.—Photomicrograph (magnification ×1200) of a supravital preparation of blood cells of a subject recently exposed to small repeated doses of radiation. Refractive neutral red bodies are seen at five-thirty and nine-thirty o'clock in the lymphocyte at the center of the print. The cluster of shadows between the two bodies are mitochondria.

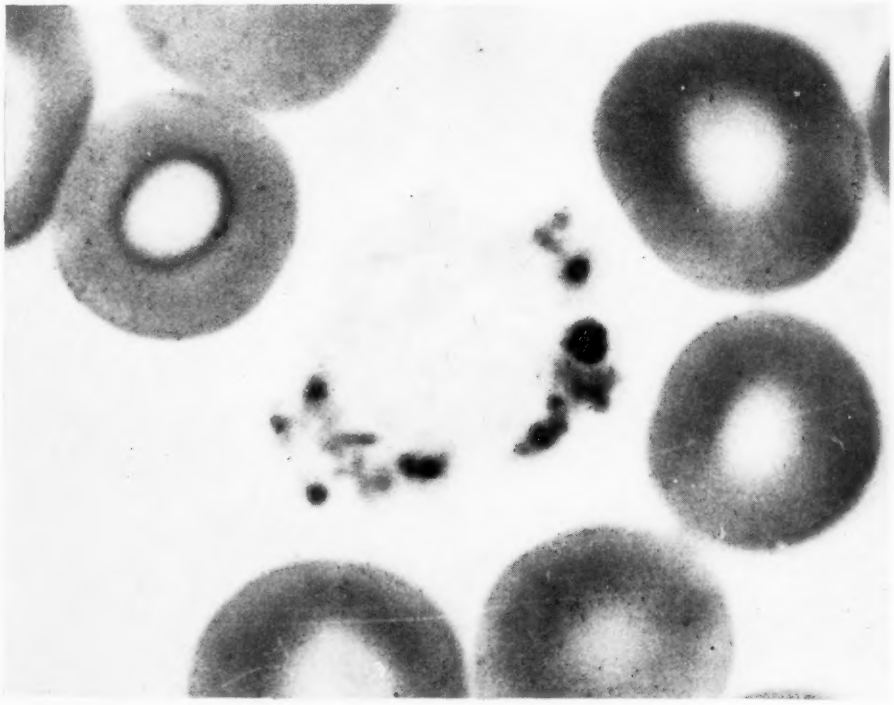

Fig. 2.—Photomicrograph (magnification ×1200) of a supravital preparation of blood from a subject after exposure to small doses of gamma rays for a period of two months. There are fifteen refractive neutral red bodies in the lymphocyte, some of which are almost out of focus. The large shadow at three o'clock is formed by two refractive bodies. The other shadows in the cytoplasm are produced by mitochondria.

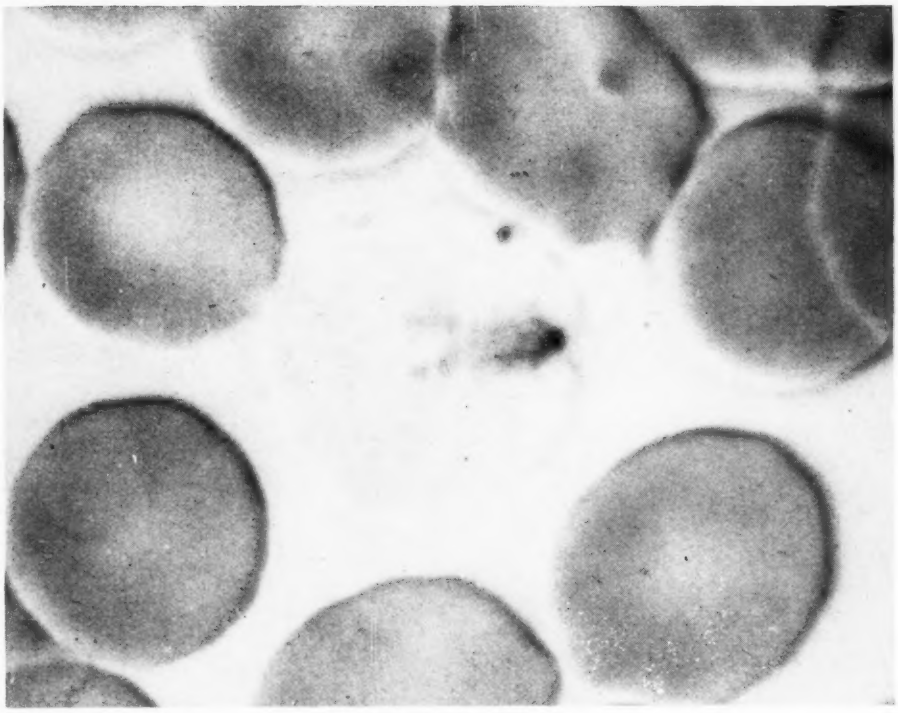

Fig. 3.—Photomicrograph (magnification ×1200) of a supravital preparation of blood cells of a subject exposed to ionizing radiation. There are fifteen refractive neutral red bodies in the lymphocyte at the center of the picture, of which only a few can be seen at this focus. One refractive body is clearly seen at one o'clock. The apex of the V shaped shadow at three o'clock is formed by a cluster of five refractive neutral red bodies. The remainder of this shadow is composed of mitochondria and three neutral red bodies, all of which are partly out of focus.

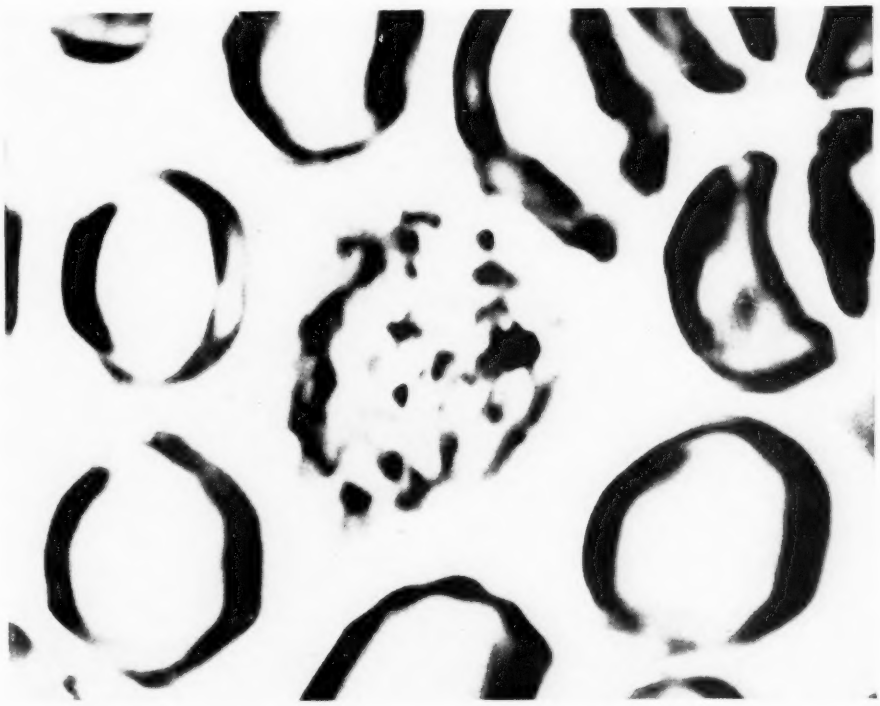

Fig. 4.—Photomicrograph of same field as shown in Fig. 3, through phase optical system (3.8 mm. oil immersion objective with integral diffraction plate, type 1B-0.25). All of the fifteen refractive bodies in the lymphocytes can be identified in this picture because of the great depth of focus of the phase microscope. Note the deep shadows cast by the cluster of refractive bodies at three o'clock and by the single body at one o'clock seen in Fig. 3. The light shadow extending from eight o'clock to eleven-thirty o'clock is produced by mitochondria.

uptake of dye by leucocytes resulting from the use of slides prepared with these strong solutions do not differ in any way from that observed in other laboratories where more dilute solutions of supravital dyes are employed.

## RESULTS

It was observed that the lymphocytes of persons exposed to ionizing radiations and toxic chemicals contained more refractive neutral red bodies than did the cells in the blood of unexposed subjects. These bodies, previously described in normal lymphocytes by Gall,[4] were irregularly placed throughout the cytoplasm. They were irregular in shape, varied considerably in size, and had a more decided brick-red color than did the neutral red-staining vacuoles (Figs. 1 to 3). There was no increase in number or size on standing twelve hours at 10 to 15° C. The number of refractive bodies per cell varied from zero to more than thirty. The refractiveness of these bodies resembles that of the granules in cells of the granulocytic series and distinguishes them from vacuoles and nonrefractive bodies which also stain with neutral red dye.

Examination of the same blood samples stained with Wright's, Giemsa's, and peroxidase stains revealed no morphologic abnormalities of the lymphocytes similar to that described.

By using contrast microscopy, additional information was gained about the structure of the refractive neutral red bodies. If individual cells in a supravital preparation were examined first through an ordinary microscope and then through a phase microscope, it was observed that the refractive neutral red bodies showed a high phase contrast while the nonrefractive and vacuolar neutral red-staining bodies showed a low phase contrast (Fig. 4). This indicates that the density of the neutral red bodies is high and that they probably exist in the solid or granular phase. The mitochondria are the only other structures in the lymphocytes that show a high phase contrast. These bodies are not easily confused with the neutral red bodies under the contrast microscope since they are smaller and their green color is poorly transmitted through the phase optical system, while the red color of the refractive bodies is well transmitted.

The increase of the neutral red bodies in the lymphocytes of the exposed groups represents essentially a quantitative rather than a qualitative change. The modification in the numbers and distribution of neutral red bodies in lymphocytes for subjects variously exposed to radiation is shown in Fig. 5. In order to facilitate the demonstration of lymphocytic changes of this nature, it was decided to differentiate between cells which contained an abnormally large number of refractive neutral red bodies and those which did not. It was agreed, therefore, on a completely arbitrary basis, to designate any lymphocyte with five or less neutral red bodies as "normal" and to consider any cell with six or more bodies as "abnormal." This terminology will be used in the remainder of this article.

One thousand sixty-four hematologic studies on 364 subjects have been analyzed. Fig. 7 is a scatter diagram showing the percentage of abnormal lymphocytes in all subjects divided into control and exposure groups. The explanation of the type and degree of exposure of each group is given in Table I.

TABLE I. TYPE AND DEGREE OF EXPOSURE OF GROUPS MENTIONED IN SCATTER DIAGRAMS

| GROUP | NUMBER OF PERSONS IN GROUP | DESCRIPTION OF WORK | DEGREE OF EXPOSURE |
|---|---|---|---|
| Group A | | | |
| Unexposed | | | |
| Class I | | | |
| Military personnel | 62 | Physical work out-of-doors | None |
| Civilian personnel | 40 | Indoor work ranging from clerical and administrative jobs to theoretical scientific work | None |
| Ambulatory clinic patients with upper respiratory infection | 8 | Varied | None |
| Class II | | | |
| Laboratory technicians and scientists | 10 | Laboratory work | None, but incidental exposure to radiation cannot be excluded completely |
| Skilled craftsmen | 49 | Plumbing, carpentry, electrical work, and machine shop work | No known exposure to radiation; plumbers exposed to lead oxide fumes within tolerance limits; machinists exposed to metal fumes |
| Group B | | | |
| External radiation | | | |
| Class I | 25 | Laboratory work | Incidental exposure to external radiation; average exposure less than 0.3 to 0.5 r per month |
| Class II | 41 | Laboratory work | Intermittent exposure chiefly to natural sources; average exposure 1 r per month or less |
| Class III | 25 | Laboratory work | Consistent exposure chiefly to radiation from accelerating equipment (cyclotron, Van de Graaff, etc.); average exposure 1.5 r per month or less; rarely did dose in any given day approach 0.1 r |
| Class IV | 22 | Laboratory work | Consistent exposure over six-month period to strong radioactive sources; monthly exposure 3.0 r or less, with certain persons exceeding this value for a single month; daily exposures frequently exceeded 0.1 r and occasionally reached 0.5 r |
| Group C | | | |
| Radioactive material A | | | |
| Class I | 8 | Laboratory work | Contact with radioactive material A, an alpha particle emitter; no deposition of material within body according to reasonably sensitive tests |

TABLE I—CONT'D

| GROUP | NUMBER OF PERSONS IN GROUP | DESCRIPTION OF WORK | DEGREE OF EXPOSURE |
|---|---|---|---|
| Class II | 17 | Laboratory work | Contact with radioactive material A; some deposition of material in the body, but average amount below maximum permissible value |
| Radioactive material B | | | |
| Class I | 29 | Laboratory work | Contact with radioactive material B, an alpha particle emitter; no deposition of material in the body according to moderately sensitive test |
| Class II | 6 | Laboratory work | Contact with radioactive material B; deposition of material in the body approaching maximum permissible amounts |
| Group D | | | |
| Uranium | 20 | Laboratory and plant work | Mild to moderate exposure to uranium chiefly as oxide fumes |

This exposure refers to the period 1943 to 1946 and does not take into consideration the previous exposure record of the subject. In practically all cases except those in Radiation Groups II and III, previous exposure to toxic chemicals or radiation was negligible. Each point in the scatter diagram represents the percentage of abnormal lymphocytes for a single subject. In the case of most of the controls, this point is based on one examination; in the case of the exposed subjects, the point represents the average of many (in one instance, thirty) examinations. Where radiation exposure was intermittent it was impossible to correlate the time of the hematologic examinations with the time of the exposure. For purposes of comparison, Figs. 6, 8, 9, and 10 show scatter diagrams of the total white blood count, the total abnormal lymphocyte count, the total lymphocyte count, and the percentage of lymphocytes in the differential white blood cell count for each exposure group. As in the case of Fig. 7, each point represents an average figure for all examinations on a single individual.

Figs. 11 and 12 show the increase in percentage of abnormal lymphocytes in persons accidentally exposed to single, large, instantaneous bursts of general body radiation. It is unfortunate that previous determinations of the percentage of abnormal lymphocytes were not made on these subjects. A complete hematologic report of these cases will be presented in a forthcoming article. Single exposures to general body gamma radiation which did not exceed 5 roentgens and which were delivered over a period of several hours produced no increase in the percentage of abnormal lymphocytes.

Similar increases in abnormal lymphocytes have been observed in rabbits and cows exposed to large doses of ionizing radiation. Morphologic changes of this type in the lymphocytes of mice and rats exposed to radiation could

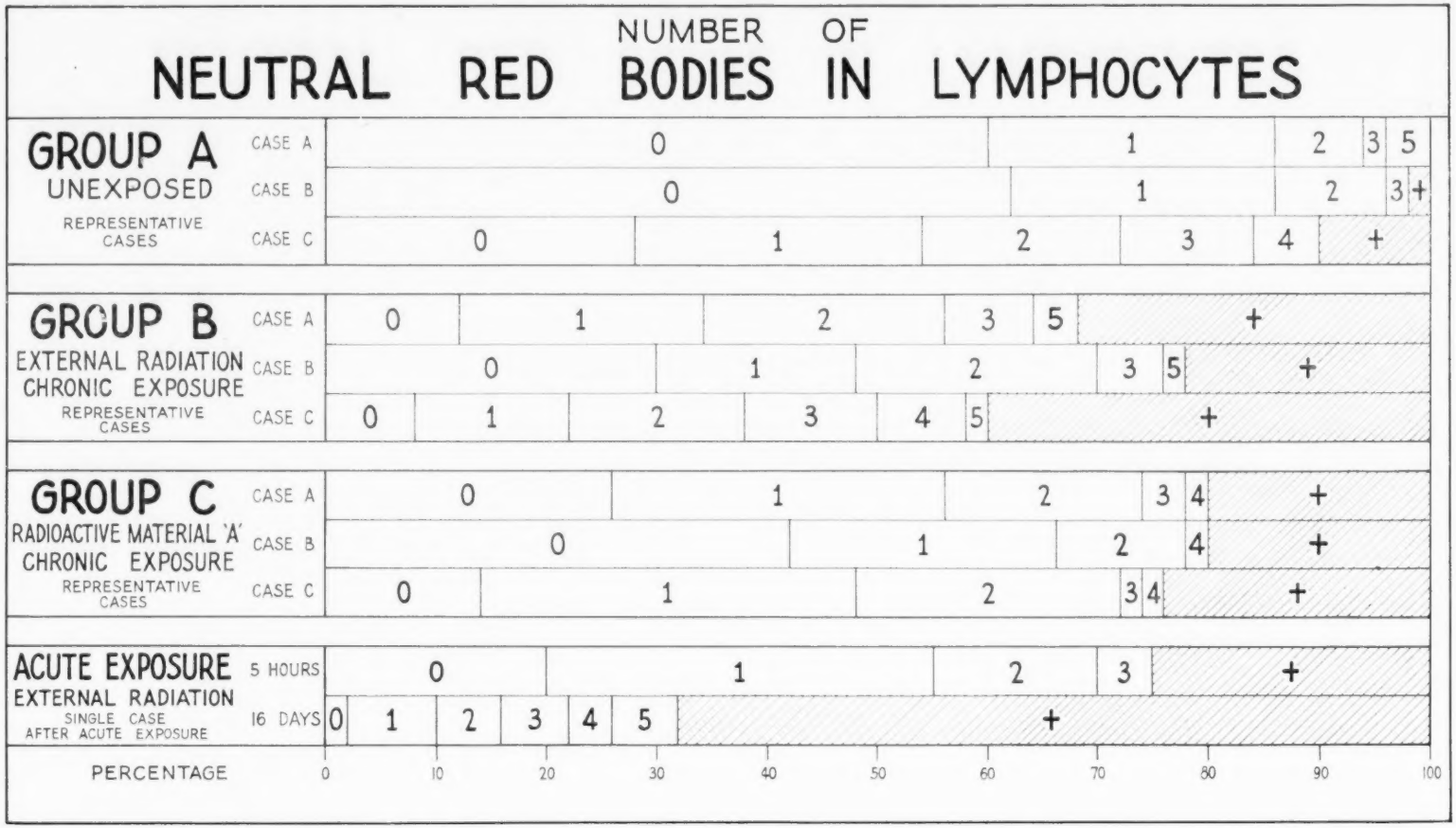


Fig. 5.—This chart arranges the lymphocytes of representative individuals in various exposure groups according to the number of refractive neutral red bodies which they contain. The bands extending across the page represent 100 per cent of the lymphocytes for an individual. The scale of lymphocytes in percentage is shown at the bottom of the chart. The numerals in each segment of a band denote the number of refractive bodies in that fraction of the lymphocytes. The lymphocytes containing more than five bodies are lumped together in one group designated by a plus sign. Thus, in Case A, Group A, 60 per cent of the lymphocytes contained no neutral red bodies; 25 per cent, one body; 8 per cent, two bodies; 2 per cent, three bodies; and 5 per cent, five bodies.

bodies; and 5 per cent, five bodies.

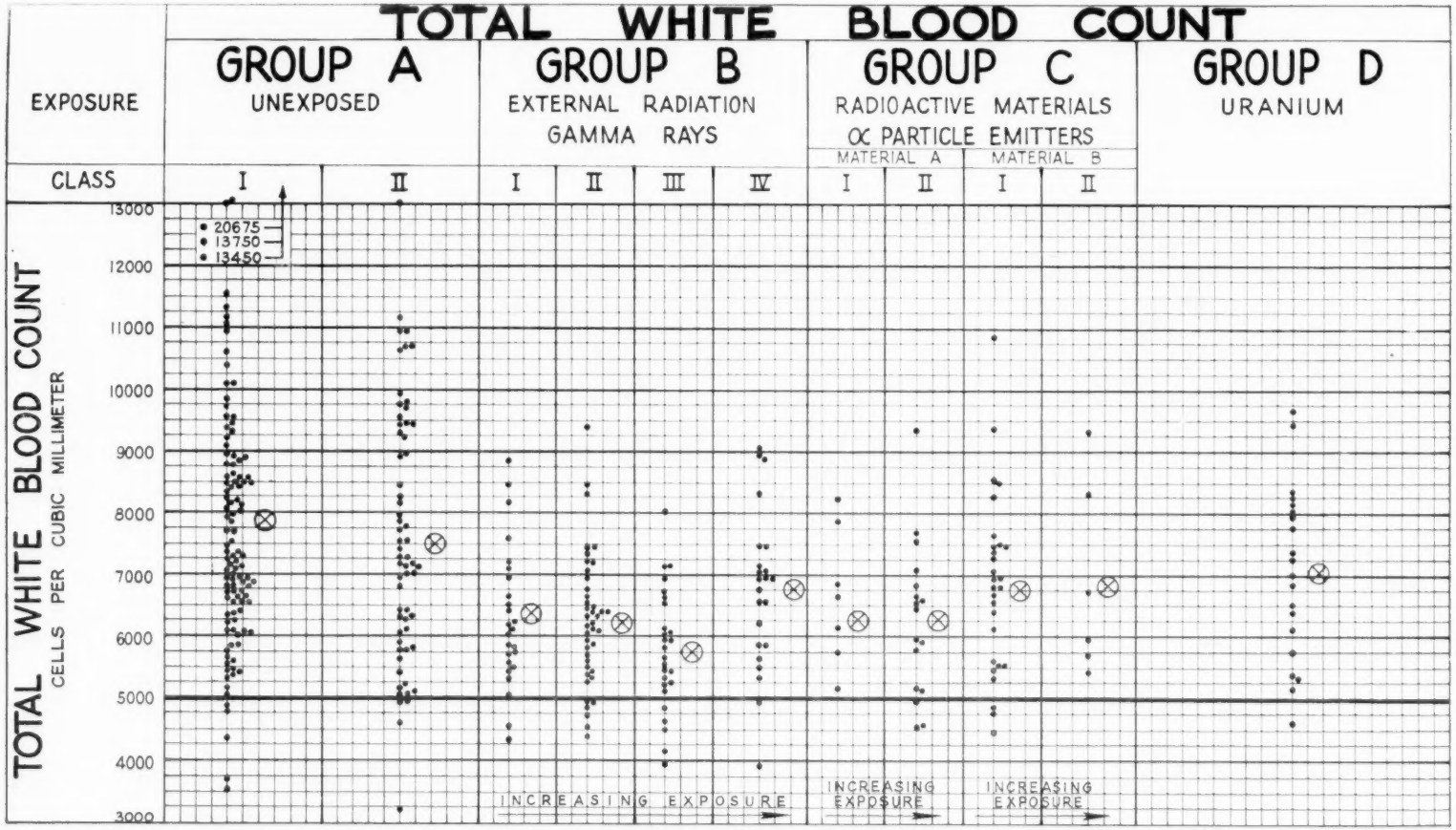


Fig. 6.—Scatter diagram showing total leucocyte count for individuals in each exposure group. The cross in each column represents the average leucocyte count for this group.

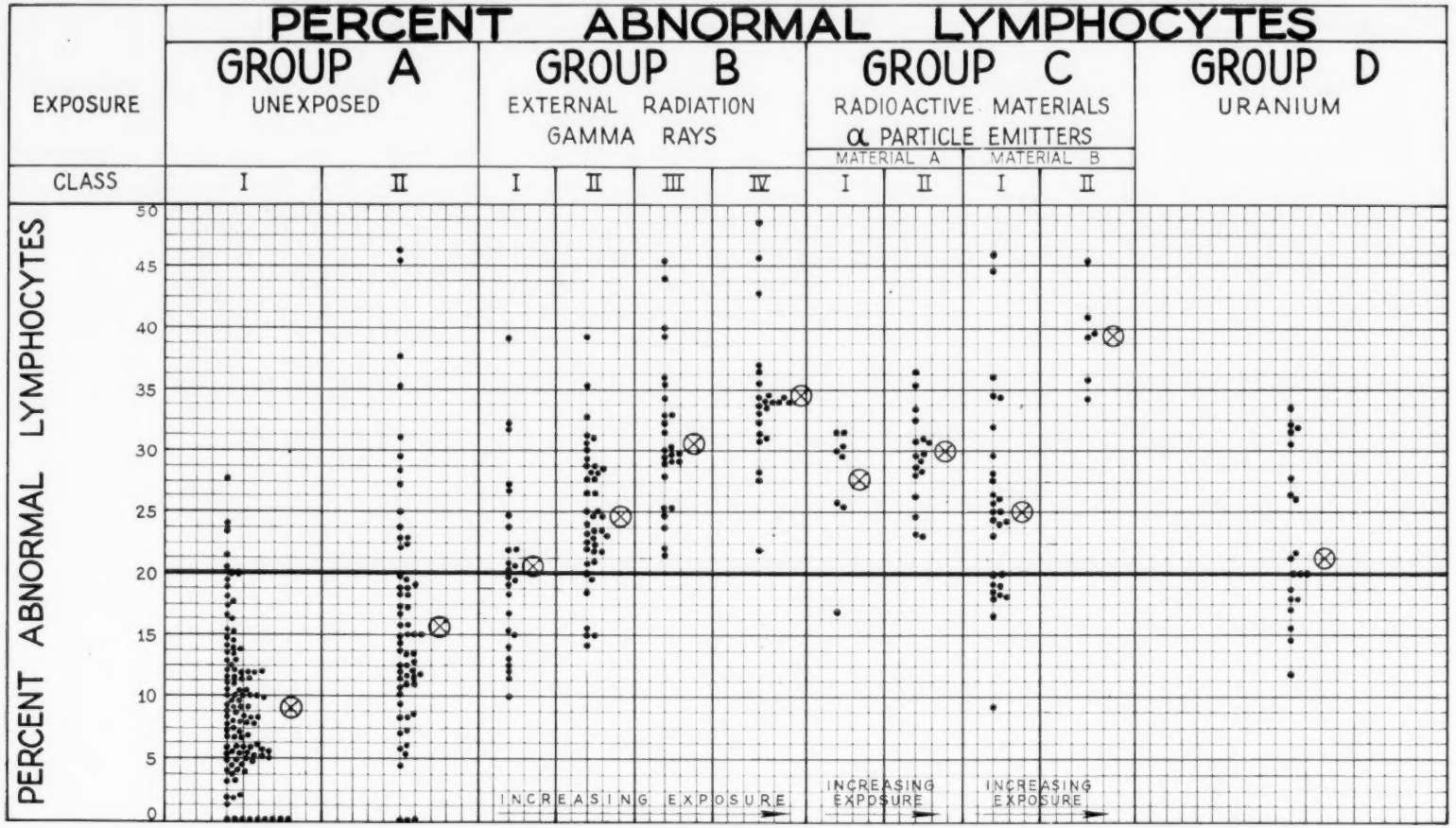


Fig. 7.—Scatter diagram showing the percentage of abnormal lymyhocytes for persons in each group. The cross in each column represents the average percentage of abnormal cells for each group.

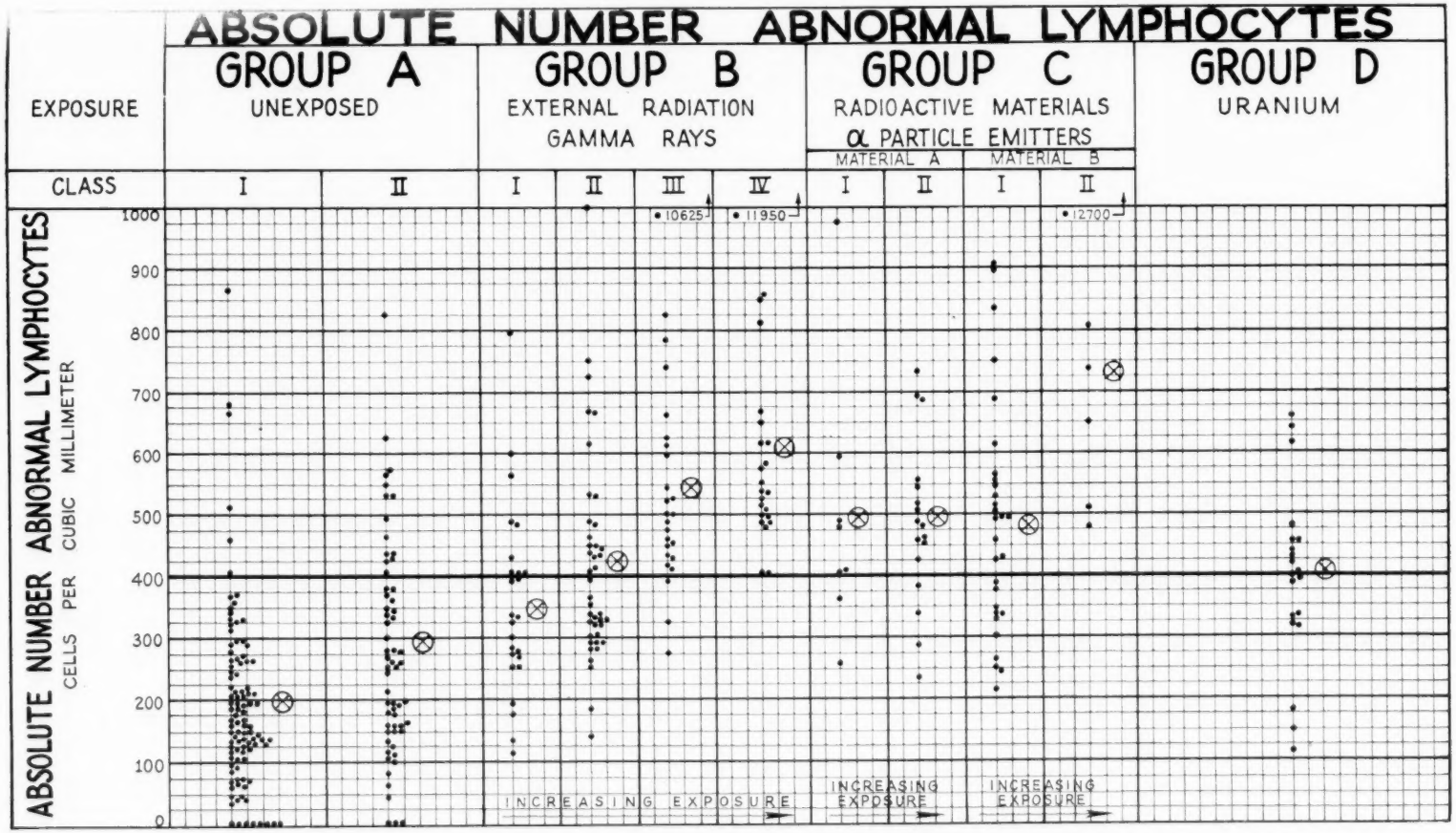


Fig. 8.—Scatter diagram showing the absolute number of abnormal lymphocytes for persons in each group. The cross indicates the average abnormal cells per cubic millimeter for each group.

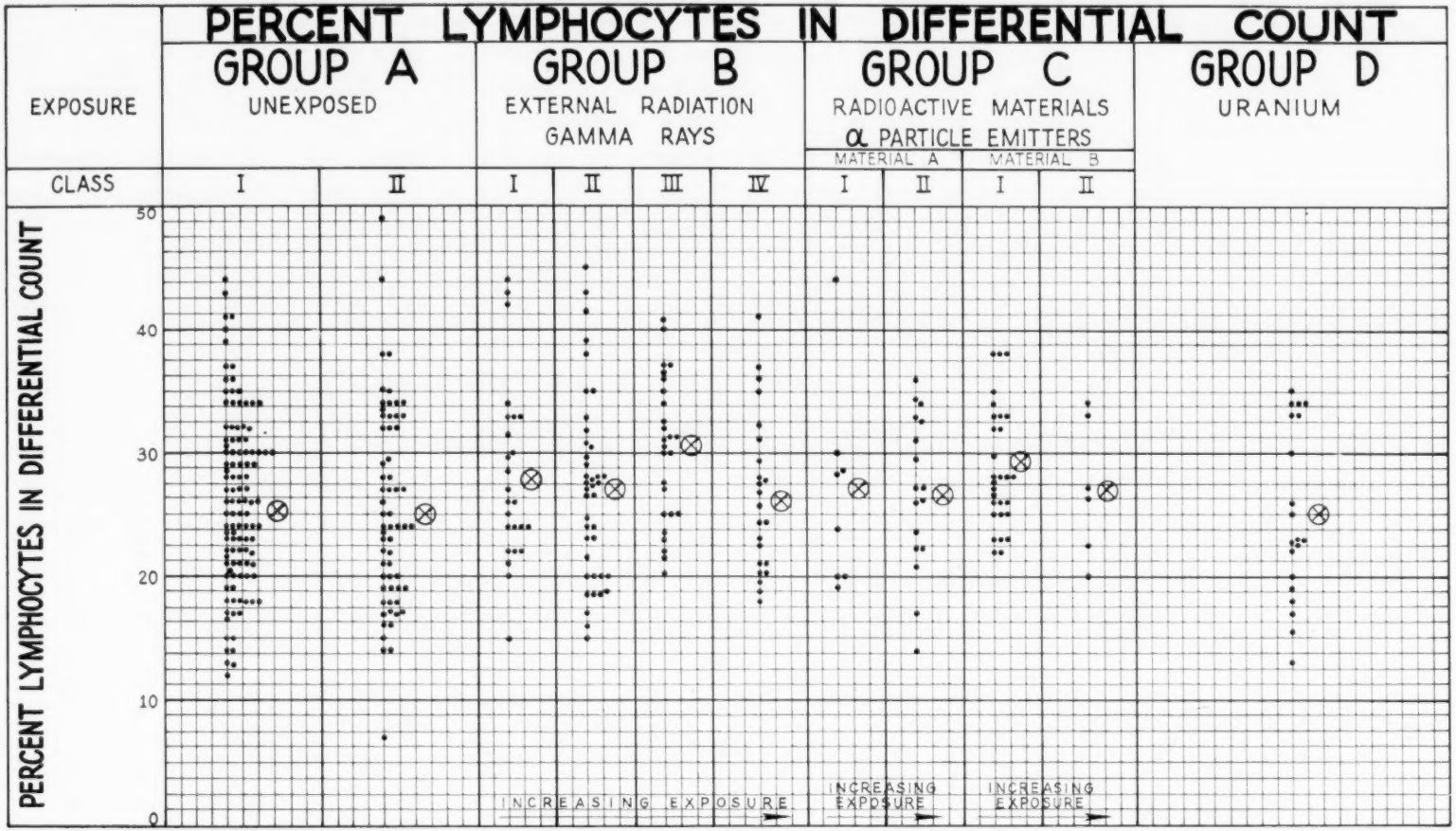


Fig. 9.—Scatter diagram showing the percentage of lymphocytes in the differential count for persons in each group. The cross indicates the average for each group.

age for each group.

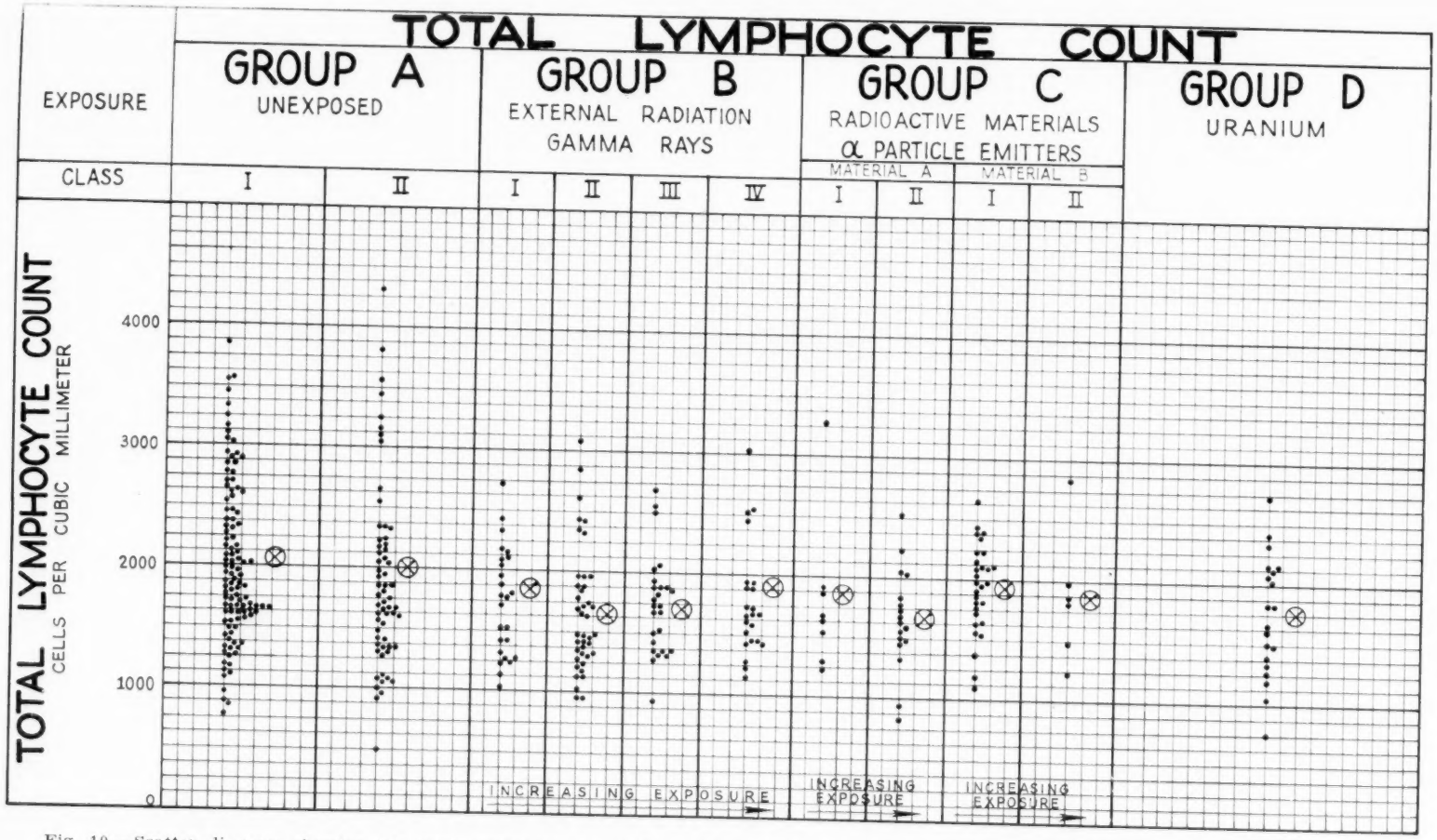


Fig. 10.—Scatter diagram showing the total lymphocyte count for subjects divided into groups. The cross denotes the average for the group.

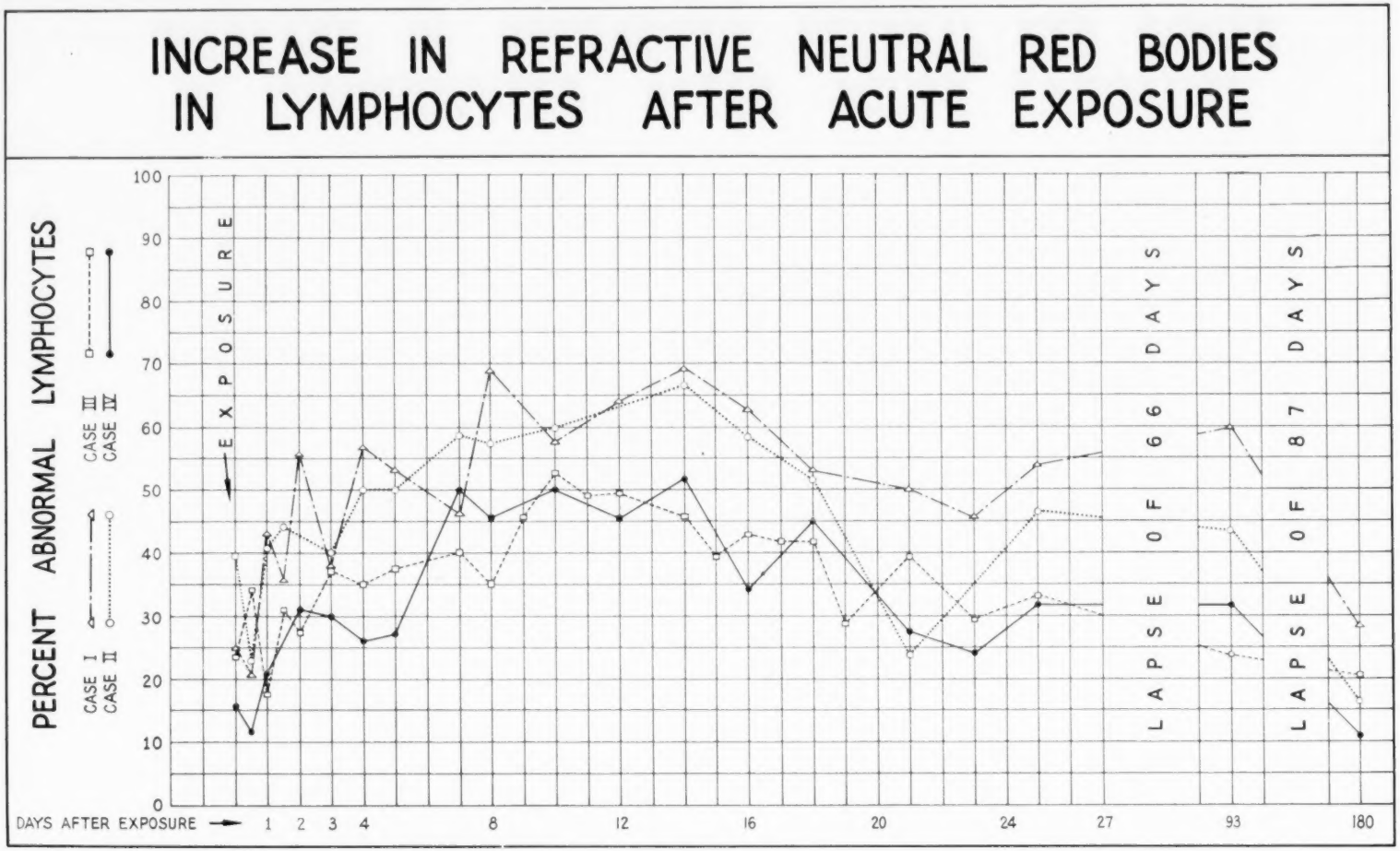


Fig. 11.—Chart showing the percentage of abnormal lymphocytes in four subjects exposed to an instantaneous burst of total body radiation. All subjects showed high initial total leucocyte counts, but subsequent changes in the blood counts indicated evidence of mild transient bone marrow injury only in Cases I and II.

injury only in Cases I and II.

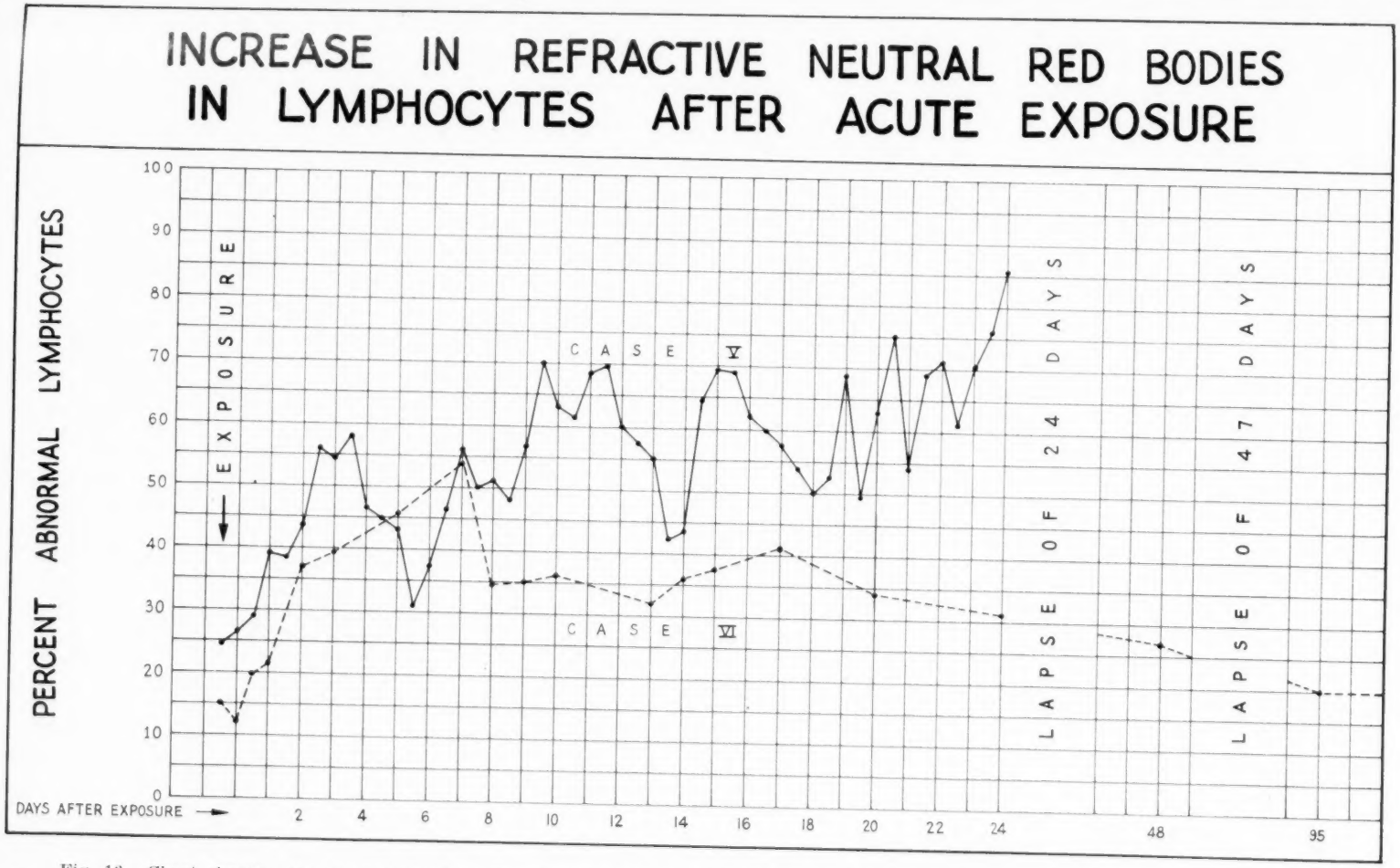


Fig. 12.—Chart showing the percentage of abnormal lymphocytes in two subjects receiving brief exposure to large doses of ionizing radiation. Case V received a lethal amount of radiation. Case VI was exposed to a relatively small dose of radiation which produced no other changes in the blood picture.

not be detected because the large amounts of neutral red dye normally taken up by the cytoplasm of the lymphocytes interferes with the identification of the refractive bodies.

## DISCUSSION

A survey of the literature has failed to disclose previous descriptions of similar morphologic changes in lymphocytes after in vivo exposure to small repeated doses of ionizing radiation. Morphologic changes in living cells of various kinds have been reported following exposure to single large doses of radiation. Prigosen[5] has reported the appearance of neutral red bodies in irradiated tumor cells. Recently Schrek[6] described an increase in cytoplasmic vacuoles in dark-field preparations of lymphocytes after in vitro and in vivo exposure to x-rays.

Inspection of the scatter diagrams in this report shows that there is an increase in abnormal lymphocytes in the persons exposed to ionizing radiation. The percentage and total number of abnormal cells can be correlated with the magnitude of chronic exposure. The significance of the data presented in these diagrams is evident without further statistical treatment. There are several features of these diagrams, however, which require further discussion.

A significant difference between the controls (Group A, Class I) and the groups exposed to radiation (Group B, Classes I, II, III, and IV) was observed only for total leucocyte count, the proportion and absolute numbers of abnormal lymphocytes. In the case of the total white blood cell count, the difference can be demonstrated by comparing the average counts for each group. Since most of the total counts of all groups lie between 5,000 and 9,000 cells per cubic millimeter, however, the value of any individual count has little significance in determining the exposure group into which the subject falls. On the other hand, the difference in the percentage of abnormal cells and absolute abnormal cell counts between control and exposed subjects is reflected in the value for an individual subject. Most of the controls have less than 20 per cent abnormal cells or 400 abnormal cells per cubic millimeter, while all of the more consistently exposed subjects show percentages and total numbers of abnormal cells above these levels. There is so little overlap of points for exposed and control groups that the appearance of few or many abnormal cells has more than chance significance in determining the exposure of any given individual.

A difference in percentage and absolute numbers of abnormal lymphocytes also exists between the controls and the groups exposed to certain chemicals. In this respect, it should be emphasized that the biologic action of natural uranium is due to its chemical rather than its radioactive properties. Thus the increase in percentage of abnormal lymphocytes in Group D is probably related to the chemical effect of uranium. The increase in percentage of abnormal cells in Group A, II (Fig. 7) is added evidence pointing to the fact that exposure to toxic chemicals changes the percentage and the absolute number of abnormal cells, since nine of the fourteen points above the 20 per cent abnormal cell level represent plumbers who were frequently exposed to lead fumes. These subjects have shown no clinical or laboratory evidence of plumb-

ism except for an occasional mild degree of basophilic stipling of the red blood cells. An increase in abnormal lymphocytes was also found in persons working with industrial nonradioactive chemicals besides uranium and lead.

It is evident from Figs. 11 and 12 that exposure to a single large dose of ionizing radiation increases the proportion of abnormal cells in the circulating blood. The response of the lymphocytes of the four subjects in Fig. 11 is essentially the same, although the radiation dosage of the subjects differed by a factor of ten. It must be concluded, therefore, that the increase in abnormal lymphocytes is not proportional to the dosage when administered as a single brief exposure.

## SUMMARY

1. Analysis of the total leucocyte counts of persons chronically exposed to ionizing radiation and toxic chemicals shows a significant statistical decrease in the exposed groups.

2. Analysis of the absolute number and of the percentage of lymphocytes in the differential counts of the same persons shows no significant change.

3. Morphologic study of supravital preparations of blood cells of persons chronically and acutely exposed to ionizing radiation indicates a striking increase in the number of refractive neutral red bodies in the cytoplasm of the circulating lymphocytes. An increase in neutral red bodies is also found in persons working with toxic chemicals.

4. These neutral red bodies have high density and may be considered to be granules. They have not been identified in fixed preparations.

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# STUDIES ON LYMPHOGRANULOMA VENEREUM: EVALUATION OF THE COMPLEMENT FIXATION TEST WITH LYGRANUM

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IN THE development of our knowledge of most infectious diseases, progress has been expedited by improvements in the methods available for diagnosis, particularly in the case of latent infections or in the frank disease when symptomatology and clinical signs are not distinctive. Thus in lymphogranuloma venereum Frei's discovery[1] of the intradermal sensitivity of infected individuals to lymphogranulomatous bubo pus constituted a great advance since it enabled a diagnosis to be made on grounds other than the clinicopathologic basis set forth by Durand, Nicolas, and Favre.[2] By means of this cutaneous reaction a variety of lesions other than those of the genitals and regional lymph nodes were linked to a common background of lymphogranulomatous infection; in some instances it has been possible later to confirm the diagnosis by the actual isolation of the etiologic agent from pathologic material.[3] Until recently, however, a serious deterrent to the routine use of the Frei test in large-scale investigations (which are highly desirable for a more complete understanding of the epidemiology of the disease) was the fact that to the great majority of clinicians there was not readily available human bubo pus of satisfactory specific activity and free from contamination with antigens derived from other infectious organisms.

Several preparations of tissues experimentally infected with virus, such as brain tissue from different animal species,[4] have been suggested and employed as a substitute for human bubo pus. These, while containing sufficient specific antigen to elicit a positive Frei reaction, also include relatively large amounts of foreign tissue which may evoke a nonspecific inflammatory response. This makes interpretation of the test difficult unless it is compared with the result of simultaneous inoculation of normal tissue emulsion as control. Furthermore, allergic hypersensitivity to the accompanying brain tissue, whether inherent in the individual or acquired only after repeated skin testing, may sometimes obscure any specific reaction to the virus antigen.[5] It should be pointed out, however, that while no control material is inoculated in tests with human bubo pus and any reaction following its introduction is taken as evidence of a positive response, there is little reason to believe that the skin of occasional individuals may not be irritated by injection of certain components of such pus irrespective of the presence of, and reaction to, lymphogranuloma antigen.

A new and improved reagent (Lygranum) for the Frei test[6] has been developed as a result of the observation that the etiologic agent of lymphogranuloma venereum will proliferate luxuriantly when introduced into the

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yolk sac of the embryonated hen's egg.[7] The enormous numbers of elementary bodies and other developmental forms of the virus[8] thus obtained have been readily separated from the bulk of the host tissue by differential centrifugation and the infective properties suitably destroyed while preserving the antigenic activity. In the experience of several groups of investigators[9] the results have been eminently satisfactory, with this purified suspension of virus showing divers features of superiority over mouse brain antigen and being at least equal in the number of positive reactors detected when compared with highly potent samples of human pus. The concentration of antigen in Lygranum preparations can be controlled, so there is no prevailing reason why it should not be available in a form even more potent than the most active specimens of bubo pus. There has been some criticism of Lygranum because of difficulty in interpreting so-called "nonspecific" reactions,[10] consisting largely of erythema without papule formation or, less frequently, small papules elicited in nonlymphogranulomatous persons. It should be emphasized again, that the zone of erythema seen at the site of inoculation of Lygranum S. T. is of no diagnostic significance whatever. Only the presence of *a definite papule of at least 6 mm.* in diameter should be taken as indicating a positive reaction with Lygranum, and *every test should be controlled* with normal yolk sac material injected simultaneously with the specific antigen. The control injection usually provokes a negligible or trivial reaction, but individuals who are sensitive to chick embryo materials may give papules with the control from normal yolk sac as large as those with Lygranum; in such persons the reaction to Lygranum S. T. should not be considered as positive.

The utility of Lygranum is not limited to the intradermal test for it has also been found potent in the detection of complement-fixing antibodies in the serum.[11] While the evidence thus far acquired has shown Lygranum to be a very sensitive indicator when used in this manner as an aid in the diagnosis of infection with lymphogranuloma virus, other studies have demonstrated that cross reactions are obtained with this antigen in complement fixation tests on the sera from patients with trachoma or inclusion blennorrhea[12] and in certain cases of atypical pneumonia or pneumonitis due to psittacosis or closely related viruses.[13, 14] This is not unexpected since, in spite of obvious differences in modes of transmission and tissue tropisms, the agents responsible for these diseases appear to share many properties, including the possession of a common antigenic factor.[14, 15] It is one of the purposes of this paper to explore the limits of the specificity of Lygranum in the complement fixation test in the light of a comprehensive summary of our experience with several hundred sera derived from persons with a variety of clinical conditions.

## REAGENTS AND TECHNIQUE

*Antigens.*—Egg-adapted lymphogranuloma virus was employed as inoculum for six- or seven-day chick embryos via the yolk sacs; the latter were harvested shortly prior to, or immediately following, death of the embryo from specific infection, all embryos being candled twice a day. Those yolk sacs, found on examination to contain abundant virus in the absence of other agents cultivable on artificial media, were pooled and ground or shaken with

ten volumes of physiologic saline. For the tests to be reported here, the virus was freed from most tissue material by differential centrifugation and finally suspended in saline containing 0.1 per cent formalin.[11] Control material was prepared from normal yolk sacs in exactly the same fashion. Nigg has found that a useful antigen may be obtained by the simple expedient of treating the crude saline suspension of virus-infected yolk sac with urea or ether.[16] Recently a combination of boiling and treatment with phenol has proved even more satisfactory[17]; not only may the specific complement-fixing activity of the antigen be enhanced thereby, but also yolk sac preparations treated in this way do not show the nonspecific fixation with sera from cases of early syphilis (see below). The mechanisms involved are not understood.

In some of the early tests we employed in place of yolk sac virus the supernate obtained by centrifugalizing at low speed a saline suspension of lymphogranulomatous mouse lungs,[18] together with control material from similarly treated normal mouse lung. The results with the two viral antigens were entirely in agreement; we preferred later to use yolk sac antigen since it was more readily available in large amounts, was usually somewhat more potent, and could readily be obtained free from contamination with other organisms. The lungs of mice may contain varying numbers of adventitious bacteria or other agents which might lead to erroneous results, although we have experienced no difficulty from this cause. Finally the handling of intranasally infected lymphogranulomatous mice constitutes a definite hazard in the laboratory.[19] The soluble antigen found in Seitz filtrates of virus-infected yolk sac or mouse lung tissue[20] has also been utilized, but this may be less potent than suspensions containing particulate virus.

For use in complement fixation tests stock antigen from any of the sources mentioned was adjusted by dilution so that it was not anticomplementary, gave definite reactions with known weakly positive lymphogranulomatous sera, and gave no reaction with sera from normal individuals.

*Sera.*—Just before use the sera were inactivated for twenty minutes at 56 or 60° C.; the higher temperature was helpful in reducing the anticomplementary behavior observed on prolonged storage of serum.[21] Serial doubling dilutions were made in physiologic saline. In most cases the lowest initial dilution of serum tested was 1:2, although on a few occasions the undiluted serum was also used. Since during fixation the serum is acting in a final dilution three times that originally introduced, we have recorded the titers as the highest final dilution giving definite specific fixation.

*Complement.*—Guinea pig serum was used, either fresh or stored at −32° C. until needed; it was titrated and diluted just before use. Two units in 0.2 ml. were usually obtained with a 1:20 to 1:30 dilution.

*Performance of Test.*—The following procedure was found to yield satisfactory results while retaining features of simplicity and convenience, permitting the test to be read within two hours after it was prepared. To a series of tubes was added 0.2 ml. of each of the reagents, in the order stated: diluted patient's serum, 2 units of hemolytic complement, specific antigen. A control series was set up in each instance with normal tissue material in place of the specific antigen. In every test controls were also included to exclude anticomplementary or hemolytic action of the antigen or patient's serum, as well as to demonstrate the activity of the complement. After incubation for seventy-five minutes in a 37° C. water bath, to each tube was added 0.2 ml. of the indicator. This volume contained a 3 per cent suspension of sheep corpuscles in saline and 2 units of amboceptor, the cells having been sensitized beforehand by incubation for thirty minutes at 37° C. Readings for fixation (inhibition of hemolysis) were made after thirty minutes longer at 37° C. Only when all controls were satisfactory was a test considered of possible diagnostic significance. The sensitivity of the test can be increased somewhat by carrying out fixation in the refrigerator overnight or by a short period of incubation in the cold following that at 37° C. Prolonged fixation in the cold increases the tendency to nonspecific fixation with normal yolk sac control material, however, so this refinement was used only rarely.

Nearly all sera were tested in serial dilutions at least twice, and the majority were tested several times to check the reproducibility of results. One additional point should be

mentioned here, namely, the desirability of testing serum specimens with reasonable promptness after their collection. If fluid sera are stored without preservative in the refrigerator for many months they frequently suffer deleterious bacterial or fungous contamination; furthermore, the specific titer tends to diminish with aging while the anticomplementary properties increase, making it difficult or impossible to test satisfactorily low dilutions of the serum. This should be borne in mind, since some sera are of low titer even when tested shortly after their withdrawal from the donor.

## PRESENTATION AND DISCUSSION OF EXPERIMENTAL DATA

1. *Tests on Sera from Persons With History or Signs of Overt Lymphogranulomatous Infection and Positive Frei Test.*—We have tested serum specimens from 149 patients falling in this classification, with the results summarized in Table I.

TABLE I. COMPLEMENT FIXATION TESTS ON SERA FROM PERSONS WITH EVIDENCE OF LYMPHOGRANULOMATOUS INFECTION

| TYPES OF LESIONS OBSERVED IN PATIENTS | NUMBER TESTED | LYGRANUM COMPLEMENT FIXATION | |
|---|---|---|---|
| | | POS. | NEG. |
| Genital and/or inguinal lesions* | 33 | 33 | 0 |
| Posterior bowel lesions† | 61 | 60 | 1 |
| Other lesions‡ | 55 | 54 | 1 |
| Total | 149 | 147 | 2 |

*This group includes early or chronic lesions of genitals and/or inguinal adenopathy.

†This group includes: (1) proctitis and/or rectal stricture; (2) other anal lesions such as sinus, fistula, etc.; (3) involvement of colon.

‡This group includes cases presenting one of the following: (1) both genitoinguinal and rectal pathology, (2) generalized systemic manifestations, (3) laboratory infections, (4) other lesions not further classified.

An analysis of the data has brought out certain points of interest. In our hands the complement fixation test gave positive results with the sera from 98.6 per cent of 149 individuals in whom the Frei reaction and history or clinical signs were indicative of overt lymphogranulomatous infection; the two failures may have been due to a very low concentration of antibodies which could perhaps have been detected by making the conditions of testing more sensitive, or to the absence of antibodies in these particular serum specimens.

The titers of the positive sera ranged from 6 to 1,920 and greater. The frequency with which various titers were encountered was as follows:

| | TITER |
|---|---|
| 32 (21.8 per cent) | 6 to 15* |
| 40 (27.2 per cent) | 30 to 60 |
| 42 (28.5 per cent) | 120 to 240 |
| 33 (22.4 per cent) | 480 or greater |

The data have been examined in an attempt to ascertain the factors influencing the complement-fixing titers obtained.

1. *Activity Status:* Usually, but not invariably, the sera of patients with clinically active lesions showed higher titers than the sera of patients with healed lesions.[22]

2. *Intensity of Frei Reaction:* The sera of individuals who yielded a large cutaneous response usually showed higher titers than those from persons whose Frei reactions were weak (see also Grace and Rake[22]). In some instances, however, patients with antibodies of

*It should be recalled that all figures concerning titer are given at threefold the usual designation in the literature. Thus most authors would give these titers as from 2 to 5.

low titer (6 to 15) gave skin reactions of greater intensity and extent than other persons whose serum titer was tenfold greater.

3. *Duration of Infection:* In recent infections the titers ranged from 6 to 960; where the disease had been in progress for three to eight years the titers observed were from 60 to 960. A patient with infection of ten years' standing was found to have a titer of 30; another whose disease dated back fifteen years showed a titer of 90, while the serum of a third individual twenty years after the onset of inguinal adenopathy showed a titer of 6. Thus the duration of infection was not necessarily reflected in the titer.

4. *Location of Lesions:* No obvious disparity in regard to antibody levels was noted between infections characterized by inguinal adenopathy and/or genital lesions and those involving the posterior regions of the bowel.

5. *Sex:* In the respective sexes the serum titers were distributed as follows:

| TITER | WOMEN | MEN |
|---|---|---|
| 6 to 15 | 8 | 24 |
| 30 to 60 | 6 | 25 |
| 120 to 240 | 15 | 24 |
| 480 or greater | 4 | 8 |

While these figures indicate that titers of 120 or greater were more common in women, the number of cases involved is too small to be statistically significant.

6. *Race:* The titers in whites and Negroes were closely parallel, that is:

| TITER | WHITES | NEGROES |
|---|---|---|
| 6 to 15 | 19 | 13 |
| 30 to 60 | 16 | 15 |
| 120 to 240 | 21 | 18 |
| 480 or greater | 7 | 5 |

7. *Concomitant Venereal Infection:* The presence of other venereal diseases did not noticeably affect the lymphogranuloma complement-fixing titer. It is noteworthy that in the group with rectal lesions (chiefly stricture and/or proctitis) thirty-five had no history or evidence of other venereal disease, twenty had signs, serologic evidence, or history of syphilis, and one had a history of gonorrhea. In the genitoinguinal group, eight had no evidence of other venereal disease, nine had a history of syphilis, and three had other venereal infections.

*Discussion:* It should be emphasized that in developing the complement fixation test with lymphogranuloma antigen, the results and technique were adjusted in an effort to set up conditions which would permit the detection of antibody in weakly reactive lymphogranulomatous sera. With the method adopted, specific fixation was obtained with the sera of 98.6 per cent of 149 clinically lymphogranulomatous, Frei-positive patients. In eleven of these the titer was only 6, and if the lowest initial dilution of serum tested had been 1:5 instead of 1:2, the demonstration of antibody would have failed. The importance of employing low as well as high dilutions of serum is thus evident, although Dulaney and Packer,[23] who in 97 per cent of their series of thirty-three clinically lymphogranulomatous patients obtained fixation with Lygranum in initial serum dilutions of 1:40 or greater, feel that a 1:20 dilution (corresponding to our titer of 60) should be considered as the lower limit for good serologic evidence of infection with the causative virus.

Several other workers have reported the results of complement fixation studies with Lygranum. Using a method similar to ours, Levine, Holder, and Bullowa[24] obtained fixation

with the sera of each of eleven known lymphogranulomatous patients and with the sera of eighteen other patients without overt lymphogranuloma venereum who yielded positive or doubtful Frei tests. Blair[25] similarly obtained fixation with the sera of four patients showing clinically characteristic lymphogranuloma and thirteen other patients without history of the disease who had positive Frei reactions. A less favorable report was published by Knott and co-workers[26] who obtained positive tests by complement fixation in 75 per cent of a group of thirty-one patients diagnosed clinically as lymphogranulomatous. From their data it is doubtful whether all of the cases in the group represented true lymphogranulomatous infection. Only 84 per cent of their patients gave positive Frei tests and 45 per cent showed positive Ito-Reenstierna tests; in some of the patients the duration of the lesions was only three days. Also contributory to the failure of Knott and associates to obtain a higher percentage of positive results may be the fact that in their serologic tests complement was used at 1:10 dilution, a concentration two to three times greater than that which we employed, which might well reduce the sensitivity of the test so that it would fail to detect weakly reactive sera such as we found in 7.4 per cent of our lymphogranulomatous series.

Howard and co-workers[27] tested seventy-six patients with positive Frei reactions; the sera of seventeen gave negative complement fixation tests. The authors state that in this group ". . . . in most instances there was a history suggesting infection with lymphogranuloma venereum in the remote past . . . . there was no clinical evidence of infection at the time the tests were made; skin sensitivity alone remained." Unfortunately the details of the serologic technique employed in their study are not stated, making it difficult to evaluate the sensitivity of the method.

Recently Florman[28] has published the results of a series of tests with Lygranum antigen, employing a modified micro-Kolmer hemolytic system. His data indicate that this method was relatively insensitive for detecting weakly reacting sera, since in a series of parallel determinations on twelve sera he obtained nine positives using the method we have recommended, while only seven showed fixation when tested by his routine technique. Furthermore, in a group of eighteen patients with positive or doubtful Frei tests he obtained only eight positives by complement fixation, a far smaller proportion than that obtained by any of the others who have compared the cutaneous and serologic tests.

2. *Correlation of Complement Fixation Titer and Serum Protein Levels in Persons With Lymphogranuloma.*—Nicolas-Favre disease is frequently accompanied by the development of hyperglobulinemia and it has been suggested[29] that this might be due to the increased production of specific antibodies. So far no direct experimental evidence has been obtained in support of the hypothesis, since the sera of lymphogranulomatous patients usually exhibit only slight virus-neutralizing properties which make quantitative evaluation difficult. It is conceivable, however, that the increased globulin values seen in lymphogranuloma reflect the production of considerable amounts of antibody which react in the complement fixation test but are not effective in virus neutralization. An opportunity to examine the problem from this angle was afforded by Dr. Marion Howard, who furnished a number of such sera together with data on their values for globulin and their albumin: globulin ratios. Table II summarizes the results of our complement fixation tests on a group of sera which were tested within a few months of their collection from the patients, together with Dr. Howard's data on the protein values. We have not included the data on several other sera which were tested after two or more years' storage in the refrigerator.

TABLE II. LYGRANUM COMPLEMENT FIXATION TITERS AND PROTEIN VALUES OF LYMPHOGRANULOMATOUS SERA

| PATIENT | COMPLEMENT FIXATION TITER | GLOBULIN | ALBUMIN : GLOBULIN |
|---|---|---|---|
| L. V. | Neg. | 1.98 | 2.36 |
| L. C. | 6 | 2.37 | 1.8 |
| L. DeL. | 6 | 2.74 | 1.56 |
| L. F. | 6 | 2.72 | 1.6 |
| W. C. | 15 | 2.42 | 1.43 |
| C. H. | 15 | 2.55 | 1.8 |
| F. G. | 15 | 2.46 | 1.8 |
| W. P. | 15 | 2.68 | 1.6 |
| H. M. | 30 | 3.26 | 1.39 |
| E. R. | 30 | 3.54 | 1.20 |
| W. M. | 60 | 2.45 | 2.0 |
| J. S. | 60 | 2.89 | 1.52 |
| M. M. | 120 | 3.15 | 1.34 |
| A. F. | 120 | 4.19 | 0.9 |
| A. C. | 240 | 5.25 | 0.64 |
| F. S. | 960 | 5.42 | 0.72 |

It was found that the globulin values showed little deviation from the normal range in those patients whose sera had only low complement-fixing titers (6 to 15), whereas in six of eight patients with serum titers of 30 or greater, the globulin content was above 3 Gm. per 100 ml. serum; the maximum globulin values were obtained with sera which showed high complement-fixing activity. Our limited data, therefore, indicate positive correlation between elevation of the serum globulin and the titer of complement-fixing antibody versus lymphogranuloma antigen in patients with Nicolas-Favre disease.

3. *Tests on Sera from Persons Infected with Certain Viruses Related to the Agent of Lymphogranuloma Venereum.*—Data previously published[12] showed that the sera of patients with trachoma fixed complement specifically in the presence of lymphogranuloma virus antigens. We have now had the opportunity, through Dr. Phillips Thygeson, to test sera from thirty trachomatous individuals. Five of the sera were anticomplementary due to prolonged storage before testing; another patient's serum gave nonspecific fixation with normal yolk sac control material. Twenty-one of the remaining twenty-four sera fixed complement specifically in the presence of lymphogranuloma antigens prepared from elementary body suspensions sedimented by high-speed centrifugation, or the soluble antigen obtained in Seitz filtrates, or both. The titers were usually low and more readily demonstrable by fixation overnight in the refrigerator. Complement fixation was demonstrable with the sera from three of four patients who had shown clinical healing under the influence of sulfonamide therapy. Despite the presence of complement-fixing antibodies in their sera the Frei test was uniformly negative whenever performed on trachomatous patients.

Sera from seven patients with psittacosis were obtained through Dr. K. F. Meyer,* who also furnished the results of complement fixation tests carried out with psittacosis antigen in his laboratory. Each of the sera showed strong complement fixation with Lygranum antigen, our titers ranging from 120 to 1920.[13]

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Complement fixation with both lymphogranuloma and psittacosis antigens was also demonstrable with each of the sera from seven patients with virus pneumonitis which was presumed or proved due to an agent related to the meningopneumonitis virus of Francis and Magill.[30] Six of these sera[13] were obtained from Dr. M. D. Eaton;* the seventh specimen was furnished by Dr. John H. Dingle.† These seven sera gave titers ranging from 6 to 60 when tested with Lygranum.

Through Dr. Thomas P. Magill‡ sera were also obtained from eleven patients with "atypical pneumonia" where there was no evidence to incriminate as etiologic agents any members of the psittacosis-lymphogranuloma group of viruses. None of these sera gave fixation with Lygranum.

*Discussion:* Complement fixation tests on the sera of patients infected with various viruses of the psittacosis-lymphogranuloma group have shown that these agents must share a common antigenic factor which stimulates the production of antibodies responsible for cross reactions within the group. The definite, albeit weak, reactions we observed when trachomatous sera were tested with lymphogranuloma antigen served to re-emphasize the close biologic relationship between these two etiologic agents which was previously predicated on the basis of striking similarities in morphology, staining, intracytoplasmic growth, and other characteristics. Unfortunately, trachoma viral antigen was not available for reciprocal tests with lymphogranulomatous sera.

Cross reactions of high titer have been demonstrated repeatedly[13, 14b, 15, 17c, 24] for human psittacotic and lymphogranulomatous sera not only when they are tested with antigens from either of these viruses, but also in the presence of antigens derived from the viruses of meningopneumonitis[30] and mouse pneumonitis.[31] Differences have been noted in the titers of individual sera versus the respective viral antigens used for testing, but these may have been due, in part at least, to varying potency of the antigens and to differences in the techniques of testing employed in the several laboratories. Smadel, Wertman, and Reagan[14b] have shown in this connection that the sera of patients with either psittacosis or lymphogranuloma venereum usually give complement fixation to approximately the same titer when tested simultaneously with standardized antigen from the heterologous or homologous virus.

The sera of various animal species infected or immunized with members of the psittacosis-lymphogranuloma group of agents show differing degrees of specificity in complement fixation tests with the several viral antigens. The situation is not entirely clear in regard to the serologic response of pigeons, which often acquire ornithosis naturally.[32a] Although the sera of birds convalescent from enzootic or epizootic ornithosis have been reported not to give fixation with Lygranum,[14b, 32b] two of three pigeon sera sent us by Dr. K. F. Meyer did, in fact, give strong fixation with this antigen (titers 120 and 480, respectively).[13] Further, Beck and co-workers[15b] found in three of four pigeons infected with the S-F strain of human pneumonitis virus a slight rise in antibodies to lymphogranuloma venereum antigen.

Several serologic studies have been carried out among persons with pneumonitis or atypical pneumonia where no direct contact with infected birds was known. Eaton and colleagues investigated two groups of such cases. In one small series an agent related to

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the viruses of meningopneumonitis and psittacosis was incriminated as etiologic agent.[33a] These patients all showed antibodies reacting indiscriminately with psittacosis, meningopneumonitis, lymphogranuloma, and mouse pneumonitis antigens; in several instances the development of a rise in titer for these antigens during the course of the illness was demonstrated.[14a] It may be noted here that in the case of another member of the psittacosis-lymphogranuloma group of viruses, namely, that causing an epidemic of severe pneumonitis in the bayou region of Louisiana, serum specimens from a few patients who had been infected showed fixation not only with antigen from the specific etiologic agent, but also with preparations from other viruses in the related group.[33b] In a second, larger series of cases of pneumonitis of unknown etiology which were studied by Eaton and Corey the sera of roughly 10 per cent of the patients showed a fourfold or greater increase in titer versus these antigens during convalescence when compared with samples collected during the acute phase.[14a] Smadel[14c] examined the sera of forty-five cases of atypical pneumonia drawn from several large urban centers of eastern United States. Ten cases were believed to be due to psittacosis; five other cases were considered on the basis of complement fixation tests possibly to be of psittacotic origin, but the sera of these patients showed no significant increase in titer during the course of recovery from the disease.

Dingle and associates,[34] in their extensive studies on atypical pneumonia, encountered six patients with respiratory disease from whom strains of virus resembling the agent of meningopneumonitis were isolated in the laboratory of Dr. Thomas Francis, Jr.* During the course of illness none of these six showed any rise in the titer of antibodies reacting with the agent of meningopneumonitis, whereas five other patients with respiratory infections, of whom only one showed atypical pneumonia, developed a fourfold or greater increase in serum titer during the course of illness. Complement fixation tests versus meningopneumonitis antigen were then carried out on the sera of a large group of well soldiers and patients with various respiratory infections. While positive reactions were only infrequently encountered among the sera from white donors, there was an appreciable concentration of positive reactions among the Negro group, irrespective of history of respiratory disease or evidence of atypical pneumonia. Since the Negroes were drawn chiefly from the South, where the incidence of lymphogranuloma is known to be high, it was suspected that the incidence of positive reactors among them might be due to crossreactions resulting from infection with lymphogranuloma virus.

Thomas and co-workers[35] made the observation that in persons with atypical pneumonia of unknown etiology sera taken during the convalescent phase frequently showed considerable complement-fixing activity versus crude mouse lung suspensions infected with any of several dissimilar viruses, including members of the psittacosis-lymphogranuloma group. These sera also reacted, although to a lesser extent, with similarly prepared suspensions of normal lungs from human beings and from mice and other laboratory animals, and with normal chick yolk sac. Acute phase sera from the same patients showed much less reactivity. It is interesting that the convalescent sera did not react with antigen of psittacosis virus propagated in tissue culture. The capacity to react with dissimilar antigens could be reduced by merely heating the sera to 60 to 65° C. or by absorption with normal mouse lung. The antigenic component responsible for fixation with atypical pneumonia sera was associated with large particles contained in the crude lung suspensions, since it could be reduced by centrifugation.

From the foregoing we conclude that: (1) antibodies reacting with Lygranum are usually demonstrable in the sera of nonlymphogranulomatous persons who are known or presumed to be infected with any one of the several other members of the group of related viruses; (2) in sera from cases of atypical pneumonia of unknown etiology, fixation versus lymphogranuloma virus is ordinarily lacking if infection with viruses of the psittacosis-lymphogranuloma group can be excluded or is highly unlikely; (3) in some cases of atypical

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pneumonia there develop during convalescence substances which react more or less nonspecifically with certain tissue components from uninfected or virus-infected lung or other tissue. These latter reactions can frequently be distinguished from specific fixation occurring with virus antigens through the use of control antigens prepared with normal tissue; they can also be reduced or eliminated by preliminary heating of the sera to 60 or 65° C., as we have done.

4. *Tests on Persons With Evidence of Syphilis.*—Complement fixation tests with Lygranum were performed on the sera of 131 individuals having evidence of syphilis. Some of the sera were specimens collected for routine premarital examinations; a large proportion was collected from patients attending venereal disease clinics. Various stages of syphilis were represented including latent, primary, secondary, and neurosyphilis. In thirty-four of the patients the syphilitic infection was congenital. Table III summarizes the results of the tests.

TABLE III. LYGRANUM COMPLEMENT FIXATION TESTS ON SERA OF PERSONS WITH EVIDENCE OF SYPHILIS

| SOURCE OF INFECTION | NUMBER TESTED | LYGRANUM COMPLEMENT FIXATION | | |
|---|---|---|---|---|
| | | POSITIVE | NEGATIVE | NONSPECIFIC |
| Congenital | 34 | 2* | 32† | 0 |
| Acquired | 97 | 59 | 30 | 8 |

*These patients were Frei-positive, married, white women.
†Thirty of these patients were also Frei tested; all gave negative reactions.

In sharp contrast to the low incidence of positive serologic or cutaneous reactions with Lygranum among those with congenital syphilis[36] are the findings in the group of individuals whose syphilitic infection was otherwise acquired. Forty-five of these ninety-seven were also given Frei tests. The findings are given in Table IV.

TABLE IV

| LYGRANUM COMPLEMENT FIXATION | FREI REACTION | NUMBER IN GROUP |
|---|---|---|
| Pos. | Pos. | 10 |
| Pos. | Doubt. | 3 |
| Pos. | Neg. | 18 |
| Neg. | Neg. | 7 |
| Nonsp. | Neg. | 6 |
| Neg. | Nonsp. | 1 |

Thirteen (29 per cent) of the forty-five persons with syphilis of noncongenital origin yielded positive or doubtful Frei tests; the sera of all thirteen fixed complement specifically in the presence of Lygranum. These individuals included five white women, five Negro men, and three Negro women; from none of them was a history of overt lymphogranuloma elicited and on examination they were asymptomatic. An additional 38 per cent of the group of forty-five gave positive complement fixation with Lygranum despite negative Frei tests.

It may be noted that the sera of eight patients with acquired syphilis showed equal fixation with normal yolk sac material and with Lygranum antigen. The significance of the various findings in the patients with syphilis as

well as in those with other genitoinfectious diseases will be discussed following the presentation of the experimental data in the next section.

5. *Tests on Sera of Persons With Genitoinfectious Diseases Other Than Syphilis.*—Complement fixation tests with lymphogranuloma antigen were performed on a limited number of sera from persons in this category, the results being summarized in Table V.

TABLE V. LYGRANUM COMPLEMENT FIXATION TESTS ON SERA OF PERSONS WITH GENITOINFECTIOUS DISEASES OTHER THAN SYPHILIS

| DISEASE | NUMBER TESTED | NUMBER POSITIVE |
|---|---|---|
| Gonorrhea | | |
| Vaginitis of childhood | 5 | 0 |
| Venereally acquired | 19 | 9 |
| Chancroid | 6 | 3 |

It will be seen that roughly the same proportion of sera from persons with venereally acquired gonorrhea or chancroid were positive versus lymphogranuloma antigen as was found for the group of syphilitic patients with acquired infection. Similarly, the failure to obtain fixation with the sera of children showing gonococcal vaginitis corresponds with the findings in the congenital syphilitic group. Frei tests were performed on the patients with chancroid; one of the three whose sera showed fixation also manifested a positive cutaneous response.

*Discussion:* The foregoing findings among the patients with syphilis and other genitoinfectious diseases are of considerable interest. Although 61 per cent of the group of ninety-seven with acquired syphilis gave positive serologic reactions with Lygranum, many of the members of the group tested were patients coming to venereal disease clinics, and we do not consider that this figure necessarily reflects accurately the incidence which one should expect to encounter elsewhere in large-scale tests on the general population. As reported previously,[11b] among the Negroes of the group the incidence was considerably higher in respect to positive reactions with Lygranum in both the cutaneous and complement fixation tests. In the work of Dulaney and Packer[23] it was noted similarly that among twenty-two Negro children with congenital syphilis no positive complement fixation reactions with Lygranum were obtained, whereas with sera from adults with neurosyphilis this serologic test was frequently positive and again the incidence of such reactions was definitely higher among Negroes than among whites.

Knott and colleagues[26] have also reported the study of a group of patients with early clinical syphilis, chiefly Negroes, attending the venereal disease clinic at the Johns Hopkins Hospital. Forty-three of the seventy-eight cases were given cutaneous tests with human Frei antigen; thirteen responded with positive or doubtful reactions. This is in surprisingly close agreement with our findings of thirteen positive or doubtful cutaneous reactions among forty-five syphilitic patients tested with Lygranum. Twenty-three of seventy-eight in the Johns Hopkins series gave complement fixation with Lygranum only; an additional thirty-four showed fixation with normal yolk sac material as well as with Lygranum. The Baltimore workers regarded as positive results all complement fixation reactions occurring with Lygranum, even when the same serum reacted equally with normal yolk sac control material. We,

on the contrary, have emphasized from the outset the difficulty of reaching any valid decision as to the specificity of a Lygranum complement fixation test when there is equal degree of fixation with the normal yolk sac control. We have considered the combined reactions as nonspecific since they are in most instances due to yolk material not removed during the preparation of the viral antigen; it is known, for example, that yolk lipids enhance the sensitivity of serologic reactions used in the diagnosis of syphilis. Our own data have included as positive reactions only those where fixation with lymphogranuloma virus antigen was marked and the reaction with yolk sac control material was entirely lacking or minimal. More recently it has been shown[17e] that many of the nonspecific reactions obtained with syphilitic sera can be eliminated through the use of antigen preparations treated with phenol and/or boiling.

Knott and co-workers[26] found that for syphilitic sera showing fixation with normal yolk sac material as well as with Lygranum antigen, absorption with Eagle flocculating antigen in many instances removed the reactivity versus both normal yolk sac control and viral antigens. A similar reduction in some cases followed antisyphilitic therapy; in other patients whose sera showed reactivity to both Lygranum and normal yolk sac control, antisyphilitic treatment was followed by a reduction in titer versus the control antigen while the titer versus Lygranum persisted. These results lend emphasis to our views as to the difficulty of assessing the specificity of fixation with Lygranum when the reaction with normal yolk sac material is also positive. Further, in certain syphilitic patients showing serologic reactivity versus Lygranum but not versus the control material, Knott and associates[26] observed that antisyphilitic treatment did not reduce the reactivity to Lygranum, nor did absorption with Eagle flocculating antigen remove the property from the serum. (See also[11b, 23].) They believed that in these latter cases at least part of the original reaction was due to specific antibody versus the virus, indicating lymphogranulomatous infection. They suggested that the results of Lygranum complement fixation in syphilitic patients could be evaluated best by repeated tests during the course of antisyphilitic treatment.

The Baltimore investigators were doubtful of the specificity of complement fixation with Lygranum because of the large number of positive tests they obtained with sera of patients giving no history or signs of overt lymphogranuloma. Koteen[37] has pointed out, however, that factual data are difficult to obtain from Negroes coming to a venereal disease clinic because these patients are prone to overlook small and painless lesions; negative histories therefore must be regarded with suspicion. Despite this, it may be noted that among thirty-four syphilitic patients of whom specific inquiry was made by Knott and colleagues, sixteen gave a history of previous genital lesions which might have been lymphogranulomatous.

Beeson and Miller[38] have recently made an important contribution to our understanding of the problem. They carried out Kahn and Lygranum complement fixation tests on 879 sera from an unselected group of white and Negro patients attending outpatient clinics at the Grady Hospital in Atlanta, Ga. The results were analyzed according to race, age, and sex. Among adult Negroes, the sera of approximately 40 per cent gave positive fixation with Lygranum and the sera of 32 per cent yielded positive Kahn tests. Among adult whites, the sera of 12 per cent gave positive fixation with Lygranum and 9 per cent gave positive Kahn tests. The age distribution of donors with positive serologic reactions followed much the same pattern for the Kahn test as for fixation with Lygranum, with a sharp rise in incidence after 14 years of age. The number of persons giving a positive test for one disease and a negative test for the other was in all groups greater than the number giving positive tests for both diseases. Furthermore, while 60 per cent of Negroes, both men and women, with positive Kahn gave positive Lygranum fixation, among the white patients with syphilis the sera of only 18 per cent of the men and 21 per cent of the women gave fixation with Lygranum, a racial difference which would not be expected if syphilis were a frequent cause of false positive serologic tests for lymphogranuloma. Beeson and Miller considered it probable that the high incidence of positive tests with Lygranum in individuals with syphilis and other venereal diseases is due to actual associated lymphogranuloma venereum infection, even in the absence of clinical findings.

From the data and discussion just presented, it appears that patients with evidence of another venereally acquired genitoinfectious disease (whether this be syphilis, chancroid, or gonorrhea) frequently show positive cutaneous and/or serologic reactivity versus Lygranum. This is in sharp contrast to the low incidence of reactions observed in patients who have acquired the same diseases by nonvenereal means. We do not believe that the high incidence of serologic reactions in persons with diseases of such diverse etiology is due to nonspecific factors but rather attribute the great bulk of the reactions to an antecedent or concomitant infection with lymphogranuloma virus even though overt clinical manifestations of the latter may be few or lacking unless very careful search is made. A small proportion of the serologic reactions observed among these patients may, however, reflect a previous infection with some other member of the psittacosis-lymphogranuloma group.

6. *Tests on Sera From Persons With Miscellaneous Respiratory and Nonrespiratory Infections.*—The data presented in the preceding sections have shown that complement fixation with Lygranum was exhibited by a high percentage of sera from (1) persons infected with agents of the lymphogranuloma-psittacosis group and (2) those with various infections of venereal origin. It seemed desirable to examine the sera of persons suffering from infections with agents unrelated to lymphogranuloma virus in which the likelihood of venereal acquisition was slight in order to determine the incidence of positive reactions in a contrasting group.

Among sixteen patients with clinically active tuberculosis, chiefly pulmonary, the sera of only two were positive versus Lygranum. One of the positive specimens was from a patient with a history of several previous attacks of gonorrhea; nothing was known about the venereal history of the second patient. The remaining donors, whose sera yielded negative results, included two with tuberculous lesions of the perianal region and genitals, respectively.

Among nine sera from patients with one of a variety of nontuberculous respiratory infections, none gave fixation with Lygranum. Similarly, in the group of eight patients listed as having various nonrespiratory infections, the complement fixation tests with Lygranum were uniformly negative (Table VI).

It is evident from the foregoing that neither the mere presence of an acute or chronic infectious process nor its localization in a site frequently affected by members of the psittacosis-lymphogranuloma group of viruses can be responsible for the findings reported in Sections 1, 3, 4, and 5 of this paper. Our failure to obtain complement fixation with lymphogranuloma antigen among the sera from the group of patients with assorted respiratory infections (Table VI), including atypical pneumonia of origin unrelated to the psittacosis-lymphogranuloma group (see Section 3), is in contrast to the findings of Florman[28] and Dulaney and Packer[23] who did obtain reactions with Lygranum C. F. in tests on sera from patients with various respiratory infections. It is possible that the difference in results may be due to the fact that we employed antigens in which the virus was separated from the bulk of host tissue materials, whereas the commercially available Lygranum used by these workers was

less refined. As pointed out previously, Thomas and co-workers[35] have shown that the sera of patients with atypical pneumonia have a tendency to fix complement in the presence of relatively crude suspensions of a variety of animal tissues.

TABLE VI. LYGRANUM COMPLEMENT FIXATION TESTS ON SERA FROM PERSONS WITH CERTAIN NONVENEREAL INFECTIONS

| PATIENT | CLINICAL CONDITION | COMPLEMENT FIXATION TITER |
|---|---|---|
| Wh. | Upper respiratory infection (Acute) | Neg. |
| P. B. | Upper respiratory infection (Convalescent) | Neg. |
| C. | Upper respiratory infection (Convalescent) | Neg. |
| H. | Pneumonia (Pn. type IX) (Acute) | Neg. |
| D. | Pneumonia (Convalescent) | Neg. |
| McK. | Pneumonia (Convalescent) | Neg. |
| M. K. | Pneumonia; pleurisy | Neg. |
| O. R. | Empyema | Neg. |
| Z. | Lung Abscess | Neg. |
| M. | Septicemia (Salmonella suispestifer) | Neg. |
| F. | Septicemia (Streptococcus hemolyticus) | Neg. |
| P. | Erysipelas | Neg. |
| S. | Osteomyelitis | <15* |
| J. B. | Lymphocytic choriomeningitis | <15 |
| A. | ? Meningitis | Neg. |
| T. | ? Escherichia coli sepsis | Neg. |
| W. | ? Dysentery | Neg. |

*Titers reported < signify that the specimens were anticomplementary at lower dilutions tested and negative at the titer indicated.

7. *Tests on Sera From Persons With Certain Organic Diseases.*—For additional information as to the range of specificity of the complement fixation obtained with Lygranum, tests were made on the sera of patients with evidence of neoplasia or cardiovascular, renal, or hepatic damage.

In the group of fourteen patients with neoplasia, the following manifestations were included: carcinoma of stomach, 2; carcinoma of sigmoid colon, 1; carcinoma of rectum, 2; papilloma of rectum, 1; rectal polyp, 2; carcinoma of pancreas, 2; carcinoma of gall bladder, 1; carcinoma of prostate, 1; lymphoma, 1; lymphosarcoma, 1. On examination one serum was anticomplementary to high titer; two other sera were anticomplementary at initial dilutions of 1:15 and 1:5, respectively, but at higher dilutions gave no evidence of specific fixation. The only positive reactions (titers 15 and 6) were obtained with sera from two men who exhibited rectal carcinoma and rectal polyp, respectively.

The fifteen patients with evidence of cardiovascular damage included individuals with congenital heart disease, various degrees of arteriosclerosis, hypertension, rheumatic heart disease, or subacute bacterial endocarditis. Only one serum, taken from a patient with rheumatic heart disease who also had a history of thyroidectomy, diabetes, and postoperative tetanus, was positive and this solely at the lowest dilution tested (titer 6).

Among ten patients with evidence of kidney damage, including glomerulonephritis, pyelonephritis, nephrosis, horseshoe kidney, pyelitis, staghorn calculus, or sulf- and methemoglobinuria, none of the sera showed specific fixation with Lygranum.

On the other hand, in a group of twenty patients with liver damage, in most of whom a diagnosis of cirrhosis was made, the sera of eight individuals gave definite fixation with titers ranging from 6 to 60 (Table VII).

TABLE VII. LYGRANUM COMPLEMENT FIXATION TESTS ON SERA FROM PERSONS WITH EVIDENCE OF LIVER DAMAGE

| PATIENT | CLINICAL CONDITION | EVIDENCE OF VENEREAL DISEASE | FREI REACTION | COMPLIMENT FIXATION TITER |
|---|---|---|---|---|
| C. | Cirrhosis | Neg. | Neg. | 15 |
| N. C. | Cirrhosis | Neg. | Neg. | 60 |
| D. | Cirrhosis | — | — | Neg. |
| G. | Cirrhosis | — | — | Neg. |
| J. | Cirrhosis | — | — | Neg. |
| K. | Cirrhosis | Neg. | — | 15 |
| R. L. | Cirrhosis | Neg. | Neg. | 15 |
| P. | Cirrhosis | — | — | <15 |
| E. S. | Cirrhosis | Neg. | — | Neg. |
| S. | Cirrhosis | — | — | 15 |
| T. B. | Cirrhosis; arteriosclerosis; heart disease | Neg. | — | 15 |
| W. C. | Cirrhosis; nontoxic thyroid adenoma | Neg. | — | Neg. |
| G. | Cirrhosis; pneumonia (Pn. type VII) | Neg. | — | 15 |
| C. M. | Cirrhosis; generalized arteriosclerosis; mild diabetes | Neg. | Neg. | 6 |
| E. O. | Cirrhosis; bronchopneumonia | Syph. | Neg. | <15 |
| R. C. | ? Cirrhosis; jaundice | Neg. | — | 15 |
| H. G. | ? Acute yellow atrophy or cirrhosis; thyrotoxicosis | — | — | 30 |
| R. G. | Miliary cirrhosis | Neg. | Neg. | <15 |
| J. B. F. | Postinfectious streptococcal hepatitis | Neg. | Neg. | <15 |
| R. L. K. | Hepatitis (? posttyphoidal) | Neg. | — | Neg. |

*Discussion:* The examination of sera from persons with cardiovascular or renal disease or neoplasia offered little or no evidence that complement-fixing antibodies versus Lygranum are developed as a result of any of these conditions per se. The positive reactions obtained with an appreciable proportion of sera from individuals showing signs of liver damage and with no overt evidence of lymphogranulomatous infection were at first rather surprising. The nature of these reactions is still not clear, and we are not in a position to draw any definite conclusion as to the antecedent history of the patients which might be responsible for this behavior of the sera. It is well known that increased globulin and decreased albumin levels may be encountered in hepatic cirrhosis, so it might be assumed that the complement-fixing reactivity of the sera with Lygranum is due to physiologic changes which are of an immunologically nonspecific character. There is also the possibility that some of the positive complement fixation reactions in this group of patients may indicate infection with some, perhaps as yet unrecognized, virus related antigenically to the members of the psittacosis-lymphogranuloma group of agents which may result in liver damage simulating that of cirrhosis.

Certain recent observations[39] are of interest in this connection. It has been possible to show that when mice are inoculated intravenously with a sufficiently large dose of the agent of mouse pneumonitis (Nigg), most of the animals will die within three or four hours. With smaller doses, however, life may be prolonged for as long as a week or more. Many of these mice become bloated and at autopsy show generalized edema, ascites, and pleural effusion as well as microscopic changes of early cirrhosis in the liver. Other observations given in the afore-mentioned studies[39] and elsewhere[40] have shown that all of the toxins of the psittacosis-lymphogranuloma group of agents cause damage to the liver cells in particular. This would support the view that some cases of human cirrhosis may be due, in part at least, to infection with an agent belonging to this group. (See also [41, 42]) Since most instances of cirrhosis, however, are undoubtedly due to other causes, the negative serologic findings with the majority of cirrhotic sera are readily explained.

8. *Tests on Sera From Laboratory Personnel.*—As part of our control series we have tested the sera of thirty-two laboratory workers, chiefly from New Brunswick personnel; the results are summarized in Table VIII.

TABLE VIII. LYGRANUM COMPLEMENT FIXATION TESTS ON SERA OF LABORATORY PERSONNEL

| HISTORY OF LYMPHOGRANULOMA VENEREUM | NUMBER OF PERSONS TESTED | COMPLEMENT FIXATION | | | FREI REACTION |
|---|---|---|---|---|---|
| | | POS. | NEG. | NONSP. | POS. |
| Negative | 28 | 0 | 27 | 1 | – |
| Equivocal | 1 | 1 | 0 | 0 | 1 |
| Laboratory infection | 5 | 5* | 0 | 0 | 5 |

*The sera of two of these workers, taken before the known infection, had been negative.

Sera from five cases of undoubted laboratory infection were examined. One was from a worker in another laboratory who developed lymphogranulomatous ophthalmitis[43]; his serum gave definite fixation with Lygranum antigen to a titer of 60. Two New Brunswick workers contracted the disease during the course of intensive animal experimentation with highly infectious material.[19] In each, the complement fixation titer of the serum rose markedly and subsided only very gradually despite the rapid alleviation of clinical signs and symptoms as a result of sulfathiazole therapy. The strongly positive Frei reactions which both workers showed in the early stages of their illness diminished in intensity during the year subsequent to infection, as shown by repeated tests during this period. Still another member of the New Brunswick personnel who was exposed to very infectious material during the frequent handling of glassware containing lymphogranuloma virus developed fever, general malaise, and pain in the region of the left shoulder due presumably to local adenopathy. In this case also, the Frei and complement fixation tests were strongly positive (serum titer 480) and administration of sulfathiazole resulted in the clearing of the general and local malaise. In the fifth individual the presence of lymphogranuloma was undiagnosed until routine complement fixation tests on the laboratory personnel revealed that his serum was strongly positive versus Lygranum. He had assisted in the care of lymphogranulomatous mice for several months and was discovered to have a history compatible with that of recent accidental infection including the development of minor herpetiform lesions on the back of one hand together with general malaise, slight fever, and headache over a

period of several weeks. His condition showed marked amelioration following treatment with sulfathiazole per os.

*Discussion:* From the foregoing observations of laboratory workers we conclude that the sera of persons in whom the likelihood of infection with agents of the lymphogranuloma-psittacosis group is minimal rarely contain complement-fixing antibodies versus Lygranum antigen. Knott and co-workers[26] obtained similar results on testing twenty white members of the hospital staff personnel as controls in their study; all of these gave negative reactions to the cutaneous and serologic tests with Lygranum.

On the other hand, cases of laboratory infection with lymphogranuloma virus, even though acquired by routes and modes other than those usual for this disease, not only become Frei positive, but also develop complement-fixing antibodies early in the course of illness, sometimes within a week after the onset; these may persist in the serum for over two years, although with the cessation of clinical activity and the possible elimination of the incitant as a result of intensive therapy with drugs of the sulfonamide group, the antibody titer tends to diminish and the Frei reaction may become completely negative again. In patients with early manifestations of the naturally acquired disease, similar reversal of the Frei reaction as a result of vigorous sulfonamide chemotherapy has been reported by others,[9d, 37, 44] although it does not commonly occur.

It may be pointed out that, as in the natural disease, laboratory infections with the virus of lymphogranuloma may vary widely in clinical manifestations. Some are mild[45] and would be unnoticed unless a Frei or complement fixation test were performed, while others may present signs of acute systemic disease.[19] It would seem a highly desirable precaution that individuals working intensively with the agent should perform complement fixation tests on their own sera at frequent intervals to check on the possibility of infection.

9. *Tests on Sera From Persons With Genitoinguinal or Lower Bowel Lesions of Questionable Origin.*—In the course of this study there was opportunity to investigate the value of the complement fixation test in affording evidence to confirm or exclude the diagnosis of probable infection with the agent of lymphogranuloma venereum in: (1) patients with genitoinguinal lesions compatible with those of early lymphogranuloma but in whom the Frei test was not definitely positive; (2) patients with genitoinguinal lesions of questionable origin but with positive Frei reaction; (3) patients with genitoinguinal lesions of questionable origin and negative Frei reaction. From Table IX it may be seen that in the small number of cases tested, complement fixation with Lygranum was regularly obtained with the sera of patients in the first two groups but with only certain sera from patients in the third group. In this latter group the positive results were obtained in those patients whose history and signs suggested the possibility of previous lymphogranulomatous infection.

In Table X are summarized the data on seventeen patients, each of whom presented one of a variety of lower bowel lesions of questionable origin. Two patients showed positive or doubtful Frei reactions, and the sera of both gave fixation with Lygranum. Among the remaining fifteen patients, the sera of only four yielded positive reactions. As might be expected, this incidence is con-

siderably lower than was observed with the sera of persons who showed bowel lesions regarded clinically as lymphogranulomatous and who also yielded positive Frei reactions (see Table I).

TABLE IX. LYGRANUM COMPLEMENT FIXATION TESTS ON SERA FROM PERSONS WITH GENITOINGUINAL LESIONS OF QUESTIONABLE ORIGIN

| GROUP | PATIENT | RACE | SEX | LESIONS PRESENTED | EVIDENCE OF OTHER VENEREAL DISEASE | FREI REACTION | COMPLEMENT FIXATION TITER |
|---|---|---|---|---|---|---|---|
| | C. A. | W | F | Inguinal adenopathy, recent | Neg. | Neg. | 120 |
| | J. G. | W | M | Inguinal adenopathy, recent plus penile lesion | Neg. | Neg. | 6 |
| a | A. H | W | M | Inguinal adenopathy, recent | Syph. | Doubt. | 120 |
| | R. L. | W | M | Inguinal adenopathy plus penile lesion | Ducrey test Pos. | Neg. | 60 |
| | R. B. | W | F | Hard mass in pelvis; ? fibrotic lymph nodes | Neg. | Pos. | 6 |
| | C. B. | N | M | Mass in right seminal vesicle, reduced with sulfathiazole | Syph. | Pos. | 6 |
| b | C. G. | W | M | Fibrosis of corpora cavernosa | Gonor. | Pos. | 6 |
| | M. J. | N | F | Bartholin's abscess | Syph. | Pos. | 120 |
| | J. L. | W | M | Balanitis | Neg. | Pos. | 6 |
| | W. W. | W | M | Nodule in scrotum | Neg. | Pos. | 6 |
| | L. B. | W | F | "Spot on womb" | — | Neg. | Neg. |
| | H. L. | W | F | Acanthosis of vulva | Neg. | Neg. | <60 |
| | V. M. | N | F | Ulcerating lesion of vulva; improved with sulfanilamide | Neg. | Neg. | 15 |
| c | M. M. | W | M | Right inguinal scar and sinus; scrotal swelling | Neg. | Neg. | Neg. |
| | O. S. | W | M | History of inguinal adenopathy; mass between rectum and peritoneum | Neg. | Neg. | 6 |
| | L. S. | Y | M | Old inguinal scars | Syph. | Neg. | 6 |

TABLE X. LYGRANUM COMPLEMENT FIXATION TESTS ON SERA FROM PERSONS WITH LOWER BOWEL LESIONS OF QUESTIONABLE ORIGIN

| PATIENT | RACE | SEX | CLINICAL CONDITION | FREI REACTION | COMPLEMENT FIXATION TITER |
|---|---|---|---|---|---|
| R. B. | W | M | Ulcerative colitis | Neg. | 6 |
| N. | — | — | Ulcerative colitis | — | Neg. |
| C. S. | W | M | Ulcerative colitis | — | Neg. |
| F. V. | W | M | Ulcerative colitis | Neg. | Neg. |
| C. E. | W | M | Stricture from hemorrhoidectomy | Neg. | Neg. |
| M. B. | W | M | Stricture from hemorrhoidectomy | Neg. | Neg. |
| H. G. | W | M | Rectal stricture | Neg. | Neg. |
| J. G. | W | M | Rectal stricture | Neg. | Neg. |
| W. B. | W | M | Pruritus ani | Neg. | Neg. |
| A. H. | W | M | Pruritus ani | Neg. | 6 |
| M. S. | W | F | Fissure in ano | Neg. | Neg. |
| H. W. | W | M | Perianal fissure | Neg. | 120 |
| E. L. | W | M | Fistula in ano | Neg. | 6 |
| C. M. | W | F | Rectal nodule | Neg. | Neg. |
| S. Y. | Y | M | Rectal polyp with histologic picture of lymphogranuloma | Doubt. | 320 |
| C. E. | W | M | ? Carcinoma of rectum in 1932; no recurrence after abdominoperineal resection | Pos. | 60 |
| R. G. | W | F | Lymphocytic granuloma of rectum | Neg. | Neg. |

*Discussion:* The limited data presented in this section indicate that the complement fixation test with Lygranum may be of definite aid in the establishment of an etiologic diagnosis in certain cases of genitoinguinal or lower bowel disease where the lack of a positive Frei reaction or the character of the lesions raises some doubt as to their lymphogranulomatous nature. In such cases repeatedly negative serologic tests would make infection with the agent of Nicolas-Favre disease seem highly unlikely, while positive tests are suggestive of an infection with some member of the lymphogranuloma-psittacosis group of agents and call for further study of the patient, including repeated Frei tests. It has been observed that the latter may subsequently become positive, thus confirming the earlier serologic findings.

10. *Tests on Sera From Persons With Urogenital Lesions of Questionable Origin.*—In Table XI are presented data concerning the findings in a group of

TABLE XI. LYGRANUM COMPLEMENT FIXATION TESTS ON SERA FROM PERSONS WITH UROGENITAL LESIONS OF QUESTIONABLE ORIGIN

| PATIENT | RACE | SEX | CLINICAL CONDITION | EVIDENCE OF OTHER VENEREAL DISEASE | FREI REACTION | COMPLEMENT FIXATION TITER |
|---|---|---|---|---|---|---|
| N. D. | W | M | Urethritis | Neg. | Neg. | 15 |
| M. F. | W | M | Urethritis | Neg. | Neg. | Neg. |
| G. | W | M | Urethritis | Neg. | Pos. | 30 |
| R. S. | N | M | Urethritis | Neg. | Pos. | 30 |
| C. S. | N | M | Urethritis | Neg. | Pos. | 6 |
| H. S. | W | F | Urethritis and cystitis | Neg. | Neg. | 6 |
| R. T. | W | F | Urethritis and cystitis | Neg. | Pos. | 15 |
| F. W. | W | M | Urethral stricture | Neg. | Neg. | 30 |
| J. D. | W | M | Urethral stricture, prostatitis and periurethral abscess | Neg. | Doubt. | 6 |
| S. P. | W | M | Urethral stricture and prostatitis | Neg. | Neg. | Neg. |
| E. N. | W | M | Urethral stricture and carcinoma of prostate | Gonor. | Neg. | 15 |
| G. | W | M | Urethral fistula | Neg. | Neg. | 15 |
| M. | — | — | Prostatic hypertrophy | Syph. | — | Neg. |
| F. B. | W | M | Cystitis | Neg. | Neg. | Neg. |
| M. C. | W | M | Cystitis | Neg. | — | Neg. |
| F. G. | — | — | Cystitis | Neg. | Neg. | Neg. |
| A. G. | — | — | Cystitis | Neg. | — | Neg. |
| J. K. | W | M | Cystitis | Gonor. | Pos. | 120 |
| A. L. | W | F | Cystitis | Neg. | Neg. | Neg. |
| L. M. | W | F | Cystitis | Neg. | Neg. | Neg. |
| M. N. | W | F | Cystitis | Neg. | Neg. | 15 |
| O. | W | F | Cystitis | Neg. | Neg. | Neg. |
| L. P. | W | F | Cystitis | Neg. | Neg. | Neg. |
| S. P. | W | F | Cystitis | Neg. | Neg. | Neg. |
| P. | W | F | Cystitis | Neg. | Neg. | Neg. |
| J. P. | W | F | Cystitis | Neg. | Neg. | Neg. |
| E. R. | N | M | Cystitis | Neg. | Neg. | Neg. |
| I. R. | W | M | Cystitis | Neg. | Neg. | Neg. |
| J. S. | W | M | Cystitis | Gonor. | Pos. | 15 |
| M. S. | W | F | Cystitis | Neg. | Pos. | 15 |
| I. W. | W | F | Cystitis | Neg. | Doubt. | Neg. |
| L. W. | W | F | Cystitis | Neg. | Neg. | Neg. |
| W. | W | F | Cystitis | Neg. | Neg. | Neg. |
| L. K. | W | M | Neurogenic bladder | Syph. | Doubt. | Neg. |
| J. R. | W | F | Hunner's ulcer of bladder | Neg. | Neg. | Neg. |
| L. E. | N | F | Hunner's ulcer of bladder | Neg. | Pos. | 6 |
| M. M. | W | M | Hematuria | Neg. | Neg. | Neg. |
| A. B. | W | M | Hematuria | Neg. | Pos. | 120 |
| J. K. | W | M | Congenital vesicorectal fistula | — | — | Neg. |

patients with urogenital lesions of questionable origin. Of eleven persons with urethral disease but with no evidence of infection by other venereal agents, four who gave positive Frei reactions and one with a doubtful Frei reaction also showed serum antibodies reacting with Lygranum antigen. In these cases the observed clinical manifestations would appear to be due to lymphogranulomatous infection; instances of abacterial urethritis believed due to this cause are already recorded in the literature.[46] Five additional individuals in the group with urethral lesions (including one with a history of gonorrhea) exhibited complement-fixing antibodies although the Frei reaction was negative. Since it is known that some cases of urethritis may be due to the virus of inclusion conjunctivitis[47] and since the sera of two persons with ocular diseases due to this agent were previously noted to give fixation with Lygranum antigen,[12] it cannot be stated which of these two related viruses may have been responsible for the urethritides in the group with negative Frei reactions.

In twenty-three patients with bladder disturbances, on the other hand, only five showed complement-fixing antibodies versus Lygranum, and four of these also gave positive Frei reactions indicating that they had been infected with lymphogranuloma virus. A more detailed account of the clinical findings in these five patients has been presented by Marshall and Endicott.[48]

## COMMENT

It seems desirable to summarize here our present information regarding the immunologic features of human lymphogranulomatous infection since this knowledge is prerequisite to the proper interpretation of the data presented in the preceding sections.

Following the introduction of active lymphogranuloma virus in the body, there is usually a relatively prompt appearance of complement-fixing antibodies which may be detected as early as a week after the time of infection[19]; during the subsequent few weeks the titer rises to a maximum, the level of which will vary with the individual. Increase in the serum globulin may also appear prior to the acquisition of cutaneous reactivity[49] which usually, though not invariably, developes after an interval of several weeks of infection.[4a, 50, 51] The clinical activity of the individual case would appear to play some, but not necessarily the major, role in determining the intensity of the cutaneous reaction as well as the serologic titer.

In the person who does not receive effective therapy the disease may regress spontaneously or may continue active for long periods of time. Even when the disease becomes clinically healed living virus may continue to reside in the tissues, perhaps dormant but still capable of stimulating the continued production of complement-fixing antibodies and enduring reactivity to the Frei test. Thus patients whose lymphogranulomatous infections date back many years will often give positive cutaneous reactions to Frei or Lygranum antigens[3, 6] as well as complement fixation with the latter[52]; they may still be infectious for their sexual partners and, moreover, active virus has in some instances been recovered experimentally from these cases of long duration.[3] The prolonged stimulation by viral antigen frequently results in the marked synthesis of complement-fix-

ing antibody, this being reflected in a high serum titer and probably also in the extent of the hyperglobulinemia so frequently noted in this disease.[42, 49, 53]

If a patient with early signs of the disease is given vigorous and adequately prolonged treatment with an effective drug such as one of the sulfonamides, the infection may be sterilized. In such case the skin hypersensitivity will wane, and over a period of months one may observe an actual reversal of the Frei reaction[9d, 19, 37, 44]; the level of complement-fixing antibodies also falls gradually, but reactions of low titer may still be demonstrable for many months after the skin reactivity has disappeared.[19] There is evidence that with clinical improvement the elevated serum globulin level may also decline toward the normal.[29, 53d, 53e]

It is well known that there may be considerable variation among individuals in their response to a given antigenic stimulus whether this is encountered in the course of clinical disease or during artificial immunization. Undoubtedly similar variation is shown in the reaction to lymphogranuloma virus, which in most patients appears to constitute a fairly potent antigenic complex. It should not be expected, therefore, that every individual who is infected with the agent will show definite cutaneous reactivity or antibody production, although the vast majority do, in fact, exhibit these responses.[54]

Are these two responses manifestations of a single immunologic mechanism or are they the result of different antigenic stimuli? It is true that some general correlation has been noted between the serum titer and the dimensions of the cutaneous reaction in lymphogranuloma[22]; this may be considered as favoring the first possibility, namely, that the positive Frei reaction depends on the presence of an adequate quantity of complement-fixing antibody in the circulation. The correlation is by no means invariable, however, and there is other evidence which would appear to favor the alternative hypothesis. In the first place, most cutaneous reactions due to the local union of antigen with circulating antibody are of the immediate variety, whereas delayed reactions are seen in the so-called bacterial allergies, wherein passive transfer with antiserum cannot be demonstrated. The Frei reaction is a delayed reaction resembling the reaction to tuberculin or chancroidal antigen. In the second place, infants born to lymphogranulomatous mothers rarely give positive Frei reactions[55] although passive transfer of complement-fixing antibodies across the placenta occurs regularly.[38, 56] Further, persons infected with other viruses of the psittacosis-lymphogranuloma group may develop potent complement-fixing antibodies which will react with Lygranum, yet fail to give positive cutaneous reactions with this antigen.[12, 57] Finally, we have obtained certain limited evidence (vide infra) which suggests that the complement-fixing antibodies may be evoked by inactive virus while the stimulus of living lymphogranuloma virus within the body is a prerequisite for the development of cutaneous reactivity.

Eight normal laboratory workers received, over a period of several months, repeated (up to ten) intradermal and subcutaneous injections of 0.25 to 1.0 ml. of formalinized Lygranum antigen, quantities many times greater than those which would be used in a cutaneous test. Their sera were tested from time to time and in two members of the group no antibodies were found; one person developed a titer of 6; three persons showed a titer of 15; two workers developed a titer of 60. None of the eight showed a positive cutaneous reaction to Lygranum, although lymphogranulomatous patients with a serum titer as low as 6 usually give a positive intradermal reaction, frequently of marked intensity. One of the workers with serum titer of 60 subsequently acquired active infection in the laboratory, following which the skin reaction became strongly positive; the serum titer also showed a further rise. The failure of Lygranum to induce cutaneous hypersensitivity in nonlymphogranulomatous individuals despite repeated injections is in agreement with the behavior of human Frei antigen under similar circumstances.[50, 51, 58]

With the foregoing in mind, we are now in a better position to consider Lygranum complement fixation in the diagnostic study of patients concerning whom the question of lymphogranulomatous infection is raised. We believe the following statements to be justified on the basis of the available data.

Among individuals with authentic lymphogranulomatous disease, irrespective of localization, nearly all will yield positive complement fixation as well as cutaneous tests with Lygranum. Only very rarely will a positive Frei reaction be obtained in the absence of serologic reactivity (see Section 1). By and large the complement fixation test, as we have performed it, appears to be definitely more sensitive and it will therefore detect an appreciable proportion of reactors who fail to yield definitely positive cutaneous tests (see Section 9). The serologic test may become positive before skin hypersensitivity has developed and may persist after the waning of the latter (see Section 8).

There are numerous patients who are asymptomatic but who exhibit definite reactivity to cutaneous and serologic tests with Lygranum. In such persons the demonstration of positive responses in both tests is strong evidence in favor of antecedent infection with the virus of lymphogranuloma, whether or not there are overt signs of Nicolas-Favre disease. Instances of this sort are not infrequently encountered among those who have acquired other venereal disease (see Sections 4 and 5) as well as among marital partners of persons who themselves present evidence of pre-existing lymphogranuloma.

In patients who show complement-fixing antibodies versus Lygranum but who do not react to cutaneous tests, the decision as to diagnostic significance requires consideration of the following possibilities. Some of these serologic reactions may reflect lymphogranulomatous infection in persons with skin anergy[50, 51, 54, 59]; it has been noted in this connection that a temporary reduction in cutaneous reactivity to Frei antigen may sometimes occur during an early syphilitic infection.[54b, 60] In certain cases cutaneous reactivity may not yet have developed or a previously existing reactivity may have faded due to sterilization of the infection while antibodies have persisted as testimony to the former presence of virus (see Section 8). In other instances, the reactions may be due to infection with a related virus such as those of ornithosis, inclusion blennorrhea, or trachoma (see Section 3). In this group of patients, therefore, the results of a single complement fixation test do not allow a dogmatic conclusion about the nature of the antecedent infection in any individual case; repeated quantitative serologic tests to determine possible alterations in titer with the passage of time, as well as a carefully taken history, may throw additional light on the etiologic background.

It was formerly believed that Nicolas-Favre disease was relatively uncommon and that it nearly always manifested itself in a frank syndrome. During the past fifteen years our knowledge of the nature and extent of the infection has been altered radically. This is due in part to the demonstration of an unexpectedly large number of clinical cases in communities where the prevalence of the disease had not been appreciated previously[4c, 61] and to the discovery of a high incidence of positive Frei reactors among those groups in the population

where promiscuity and poor sexual hygiene frequently eventuate in other venereal infections.[4d, 61] The results of complement fixation tests with Lygranum have extended the data previously obtained from cutaneous tests. There is now abundant evidence that the natural history of lymphogranulomatous infection is in some respects not unlike that of tuberculosis or syphilis. All are highly contagious, but in many individuals the infections are so mild that they pass unnoticed unless thorough physical examination and history are obtained; other persons experience subclinical or asymptomatic infections which are only detected by immunologic methods. With the limitations previously noted, the complement fixation test with Lygranum is a valuable epidemiologic tool, since it can serve as a screening device for the selection of those elements in the population which might most profitably be further investigated.

## SUMMARY

1. In a group of 149 patients who showed a wide variety of overt lesions regarded clinically as lymphogranulomatous and who gave positive cutaneous reactions to lymphogranuloma antigens, the sera of over 98 per cent were found to fix complement in the presence of Lygranum.

2. The antibody titers varied markedly among individuals; differences as great as several hundredfold were observed. It was noted that with the technique employed approximately 10 per cent of the lymphogranulomatous sera reacted, but weakly, giving definitely positive results only in the lowest serum dilution tested (titer 6 by our notation).

3. The limited data available suggested a correlation between the titers of complement-fixing antibodies and the globulin values of lymphogranulomatous sera.

4. Complement-fixing antibodies versus Lygranum were also demonstrated regularly in the sera in several cases of accidental laboratory infection with the virus and in the sera of groups of Frei-positive persons (1) who showed evidence of urogenital localization of lymphogranuloma, or (2) who showed lesions of questionably lymphogranulomatous origin, or (3) who appeared asymptomatic in regard to lymphogranuloma venereum.

5. In healthy individuals (for example, laboratory workers) who were not known to have had contact with viruses of the lymphogranuloma-psittacosis group, the complement fixation test with Lygranum gave negative results. In contrast, the sera of patients who were known to have been infected with other members of this group of related viruses in most instances gave reactions with lymphogranuloma antigens.

6. Reactions were only rarely observed among patients suffering from nonvenereal infectious diseases due to agents unrelated to the lymphogranuloma-psittacosis group, irrespective of the site of localization.

7. The sera of patients with neoplasia or cardiovascular or renal disease seldom showed reactions with Lygranum. An appreciable proportion of the sera from a group of patients with evidence of hepatic damage did show such complement fixation; the possible significance of this is discussed.

8. The results of complement fixation tests with Lygranum supported the concept that among those who acquire other genitoinfectious diseases as a consequence of sexual promiscuity there may frequently occur a concomitant infection with the virus of lymphogranuloma venereum.

9. The analysis of the available data indicates that the complement fixation test with Lygranum has certain limitations but may properly be considered useful in diagnostic and epidemiologic studies.

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# COAGULATION TIME OF BLOOD HEPARINIZED IN VITRO: CORRELATION OF RESULTS WITH THOSE OF THE HEPARIN TOLERANCE TEST

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RECENTLY we reported on a heparin tolerance test[1] and gave results obtained in a study of normal subjects and patients suffering from various types of intravascular thrombosis. This paper is presented to show the results obtained with a test of coagulation time of whole blood heparinized in vitro and to compare these results with those obtained with the more complex heparin tolerance test.

The idea of determining coagulation time after adding heparin to blood in vitro rather than in vivo was suggested by papers published by Waugh and Ruddick.[2, 3] These workers used a series of test tubes containing progressively increasing amounts of heparin, each of which contained 1 ml. of venous blood. After the coagulation time of the blood in each of the tubes was determined, a curve was charted and from this they derived their conclusions. We wished to establish a simpler procedure.

If the blood volume of an average normal adult were 5 liters, 25 mg. of heparin given intravenously for a heparin tolerance test would produce a concentration of 0.005 mg. of heparin per cubic centimeter of blood ten minutes after injection, at which time the blood and heparin should be mixed completely. Arbitrarily then, we decided to determine the coagulation times of 1 ml. of blood drawn from normal persons and patients suffering from intravascular thrombosis when the blood was added immediately after withdrawal to 0.005 mg. of heparin. Obviously, after an intravenous injection of 25 mg. of heparin the concentration of heparin is not always 0.005 mg. per milliliter of blood, since the blood volume of adult individuals varies. Hence, a slight discrepancy might be expected in comparing the coagulation times of blood obtained in the heparin tolerance test and in the test of heparinized blood in vitro.

The coagulation test of blood heparinized in vitro was performed as follows. Three 10 by 75 mm. glass tubes were used for each test. One tube was left empty. In the second tube, 0.5 ml. of a 0.9 per cent solution of sodium chloride without heparin was placed. In the third tube, 0.005 mg. of heparin dissolved in 0.5 ml. of 0.9 per cent solution of sodium chloride was placed. Blood was withdrawn from the antecubital vein and 1 ml. was measured carefully and placed in each of the three tubes. The second and third tubes were vigorously inverted three times as soon as the blood was added in order to mix the blood

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TABLE I. COAGULATION TIME IN MINUTES OF TWENTY-SIX NORMAL SUBJECTS

| SUBJECT | AGE | SEX | WEIGHT | 1 ML. OF BLOOD | 1 ML. OF BLOOD AND 0.5 ML. 0.9 PER CENT SODIUM CHLORIDE | 1 ML. OF BLOOD, 0.5 ML. 0.9 PER CENT SODIUM CHLORIDE, AND 0.005 MG. HEPARIN | 1 ML. OF BLOOD 10 MIN. AFTER INTRAVENOUS INJECTION OF 25 MG. HEPARIN |
|---|---|---|---|---|---|---|---|
| 1 | 36 | F | 128 | 5.5 | 6.0 | 64 | 61 |
| 2 | 38 | F | 127 | 5.5 | 5.5 | 58 | 53 |
| 3 | 20 | F | 120 | 6.0 | 6.0 | 51 | 46 |
| 4 | 47 | F | 153 | 4.5 | 5.0 | 55 | 47 |
| 5 | 19 | F | 129 | 6.5 | 6.5 | 93 | 82 |
| 6 | 25 | F | 126 | 4.0 | 4.0 | 37.5 | 54 |
| 7 | 27 | F | 112 | 5.0 | 5.5 | 45 | 54 |
| 8 | 20 | F | 144 | 6.5 | 6.5 | 63 | 51 |
| 9 | 21 | F | 125 | 8.0 | 9.0 | 65 | 63 |
| 10 | 34 | F | 138 | 9.0 | 8.5 | 77 | 65 |
| 11 | 46 | F | 162 | 7.5 | 8.0 | 75 | 66 |
| 12 | 38 | M | 154 | 6.5 | 5.5 | 84 | 72 |
| 13 | 48 | F | 143 | 8.0 | 7.5 | 62 | 66 |
| 14 | 25 | F | 122 | 7.5 | 7.0 | 58 | 53 |
| 15 | 22 | F | 110 | 7.0 | 7.5 | 61 | 57 |
| 16 | 33 | F | 128 | 9.5 | 9.0 | 82 | 68 |
| 17 | 28 | F | 117 | 6.0 | 6.0 | 47 | 56 |
| 18 | 24 | F | 124 | 6.0 | 7.5 | 54 | 46 |
| 19 | 28 | F | 116 | 9.0 | 8.5 | 44 | 66 |
| 20 | 58 | F | 134 | 8.0 | 7.5 | 59 | 81 |
| 21 | 24 | F | 138 | 12.0 | 13.0 | 55 | 52 |
| 22 | 24 | F | 118 | 7.0 | 8.0 | 52 | 59 |
| 23 | 45 | F | 148 | 9.0 | 9.0 | 72 | 63 |
| 24 | 60 | M | 182 | 6.0 | 6.5 | 46 | 53 |
| 25 | 29 | F | 132 | 6.0 | 6.0 | 50 | 57 |
| 26 | 41 | M | | 11.0 | 10.0 | 76 | 64 |

TABLE II. COAGULATION TIME IN MINUTES IN NINETEEN CASES OF THROMBOSIS

| SUBJECT | AGE | SEX | WEIGHT | 1 ML. OF BLOOD | 1 ML. OF BLOOD, 0.5 ML. 0.9 PER CENT SODIUM CHLORIDE, AND 0.005 MG. HEPARIN | 1 ML. OF BLOOD 10 MIN. AFTER INTRAVENOUS INJECTION OF 25 MG. HEPARIN |
|---|---|---|---|---|---|---|
| 27 | 38 | M | 149 | 6.0 | 24 | 18 |
| 28 | 51 | M | 184 | 6.5 | 21 | 22 |
| 29 | 56 | M | – | 8.0 | 36 | 44 |
| 30 | 65 | M | 128 | 9.0 | 98 | 74 |
| 31 | 57 | M | 180 | 7.5 | 14 | 11 |
| 32 | 57 | M | 135 | 5.0 | 8 | 10 |
| 33 | 39 | M | 129 | 5.5 | 58 | 52 |
| 34 | 53 | M | 146 | 7.0 | 60 | 68 |
| 35 | 28 | M | 153 | 8.0 | 64 | 59 |
| 36 | 50 | M | – | 8.5 | 33 | 37 |
| 37 | 40 | M | 190 | 6.5 | 51 | 56 |
| 38 | 44 | M | 163 | 6.0 | 51 | 46 |
| 39 | 34 | M | 118 | 7.0 | 75 | 69 |
| 40 | 38 | M | 165 | 6.5 | 10 | 11.5 |
| 41 | 33 | M | 140 | 7.0 | 10 | 9 |
| 42 | 38 | M | 151 | 6.0 | 16 | 10 |
| 43 | 33 | M | 140 | 6.0 | 32 | 43 |
| 44 | 38 | M | 151 | 5.5 | 31 | 34 |
| 45 | 33 | M | 160 | 8.0 | 35 | 40 |

TABLE III. REPEATED COAGULATION TIMES IN MINUTES ON DIFFERENT DATES IN THREE NORMAL SUBJECTS

| SOLUTION TESTED | SUBJECT 1 AGE, 38; SEX, F; WEIGHT, 122 | | | | | SUBJECT 2 AGE, 18; SEX, F; WEIGHT, 131 | | | | SUBJECT 3 AGE, 26; SEX, F; WEIGHT, 117 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | FEB. 22 | MAR. 5 | MAR. 9 | APR. 1 | APR. 11 | FEB. 22 | MAR. 5 | APR. 1 | APR. 11 | FEB. 22 | MAR. 5 | MAR. 9 | APR. 11 |
| 1 ml. of blood | 4.5 | 6.5 | 7.5 | 5 | 5.5 | 5.5 | 6 | 5 | 5.5 | 7 | 7 | 7 | 7 |
| 1 ml. of blood and 0.5 ml. 0.9 per cent sodium chloride | 5 | 6.5 | 8.0 | 5 | 6 | 5.5 | 6 | 5.5 | 5.5 | 7 | 8 | 7.5 | 7.5 |
| 1 ml. of blood, 0.5 ml. 0.9 per cent sodium chloride, and 0.005 mg. heparin | 50 | 54 | 51 | 56 | 48 | 56 | 57 | 54 | 52 | 59 | 51 | 59 | 46 |
| 1 ml. of blood 10 min. after intravenous injection of 25 mg. heparin | 56 | 63 | 54 | 60 | 64 | 51 | 59 | 48 | 64 | 48 | 44 | 52 | 57 |

with the solutions. The timing was begun as soon as the blood was drawn from the vein into the syringe. All tubes were inverted at thirty-second intervals until clotting occurred as in the heparin tolerance test. All tests were carried out at hospital room temperatures. Inasmuch as some question arose concerning loss of potency of the dilute solution of heparin used in the test, a fresh solution of the heparin (10 mg. in 1,000 ml. of 0.9 per cent solution of sodium chloride) was made every seven days or oftener as needed.

The coagulation time of blood heparinized (0.005 mg. per milliliter) in vitro was determined, and the heparin tolerance test was carried out successively on the same day in forty-five persons. The results of the two tests are compared in Tables I and II.

The coagulation time of 1 ml. of blood did not differ significantly from that of 1 ml. of blood mixed with 0.5 ml. of 0.9 per cent solution of sodium chloride (Tables I and III).

In this study the coagulation times of blood heparinized in vitro and in vivo were essentially similar for each of the persons who were tested. Relatively slight differences may be explained by variations in blood volumes from 5,000 ml. in different individuals and also are probably within the limits of the experimental error inherent in either method.

The results of the heparin tolerance test can be closely duplicated by testing the coagulation time of blood added to heparin in vitro immediately after withdrawal if the concentration of heparin is approximately the same. The coagulation time of heparinized blood in vitro is a simpler test.

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# A NEW METHOD FOR THE INDIRECT MEASUREMENT OF BLOOD PRESSURE IN THE RAT

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VARIOUS methods for the indirect measurement of blood pressure of the rat have been reported in the literature[1-3] and summarized in *The Rat in Laboratory Investigation*.† It has become common practice to determine the blood pressure of the rat by the method of Williams, Harrison, and Grollman.[3] The divergent and inconsistent results obtained by comparison of both direct and indirect blood pressure measurements led Schroeder[4] to doubt the validity of the various indirect methods of measurement of blood pressure in the rat. Shuler, Kupperman, and Hamilton[5] attributed these discrepancies to the size of the sphygmometer cuff applied to the base of the tail and to the sensitivity of the plethysmograph. By changing the membrane of the plethysmograph to a much finer membrane, and by making the size of the oncometer tube attached to the plethysmograph much smaller (½ mm.), they were able to increase the sensitivity of the instrument. However, these workers did not give any blood pressure values for unheated, unanesthetized normal animals obtained with these changes, although many values were reported for the anesthetized normal animal. Proskauer, Neumann, and Graef[6] studied independently the problem of these discrepancies. Using the apparatus of Williams and co-workers,[3] modified so as to be in agreement with the changes recommended by Shuler and associates,[5] they came to the following conclusions. "The systolic pressure of trained, normal unanaesthetitized rats, as measured by a modification of the indirect method in which there was no change in the rectal or cutaneous temperature, was 65 to 95 mm. Hg at room temp. 24-28° C." These blood pressure values were still inconsistent with those of direct puncture, although Shuler and co-workers got higher values by the indirect method which agreed with the direct method when the rats were anesthetized.

Since we had been aware since 1943 of the discrepancy between the direct and indirect methods of blood pressure measurement, we also independently sought methods by which these disagreements could be explained. On the assumption that the tail method of Williams and co-workers was fundamentally sound, we attributed our inability to use the method to the relative inability of the described apparatus to measure the small volume changes taking place in the tail before and after constriction of the sphygmometer cuff. To overcome this obstacle, we resorted to a very sensitive photoelectric system by which the change in volume of the tail was measured by the change in the size of a slit formed between a metal knife-edge adjusted to form the slit by nearly touching the upper surface of the tail. The distance of the upper surface of the tail from

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†Griffith, John Q. Jr., and Farriss, Edmond J. (editors): The Rat in Laboratory Investigation, Philadelphia, 1942, J. B. Lippincott Co.

the metal knife-edge depends on the total volume of the tail which changed on lowering or raising the pressure in a sphygmometer cuff above or below the systolic pressure. Even with this extremely sensitive system the necessity of heating normal unanesthetized animals in order to obtain volume changes in the tail was not completely overcome. The results obtained[7] were very much in agreement with those of Proskauer and co-workers. During the course of our experiments, it was observed that:

1. Even with our more sensitive method of recording volume changes in the tail, we were generally unable to obtain volume changes without heating the animal.

2. As the rats were warmed, readings could gradually be obtained, the volume change being greater as more heat was applied. However, these volume changes did not occur at the same sphygmometer pressure-cuff pressure but at higher indicated pressures. There seemed to be a proportionality between temperature and observed blood pressure reading.

3. When the rats were heated excessively the blood pressure reading fell suddenly to very low levels.

4. In attempting to standardize the rate of heating, different rats responded differently to the same heating conditions, and the same rat responded differently on different days to given heating conditions.

5. There were no optimal conditions under which comparable blood pressures were obtained in the same rat or in different normal rats.

6. Rats which gave no indirect readings at normal room temperature, when nembutalized, gave indirect blood pressure readings at normal room temperature and higher readings when heated under anesthesia.

For these reasons, it was obvious that a more dependable method of indirect blood pressure measurement would have to be developed if the rat were to be used for chronic blood pressure studies. This method would have to fill the following prerequisites:

(1) The indirect method of determining the blood pressure must be on some appendage other than the tail, in order to obviate the "reflex vasoconstriction" of the arteries of the tail which was influenced by heating or anesthetizing the animal.

(2) The normal, unheated, unanesthetized animal must be used in order to eliminate all contributing factors which would influence blood pressure.

(3) In taking the blood pressure, control of all physical factors (light, handling, type of restraint, etc.) which would affect blood pressure must be possible.

After approximately one and one-half years of further study an instrument and a technique was developed which met the stated requirements. It depends on (1) use of the foot (Griffith's technique),[1] and (2) measurement of volume change (Byrom and Wilson,[2] and Williams and associates[3]) of the foot (Griffith) by means of the change in volume and intensity of light as measured by a photocell-microammeter arrangement.

The instrument and technique which have eventually given us the mentioned prerequisites are described below.

*The Rat Holder.*—The restraining cage has been developed with a view to comfort and darkness (for quietness); it is well ventilated and so constructed as to leave the two hind legs suspended freely at the groin. The "head" part of the cage is of metal with a large overlapping cover. This cover is not airtight and permits free entrance of air but not light. At approximately the chest region of the rat a leather "body holder" is fixed to the metal head case with a clamp. This leather is split along the "back." At the "tail end" of the cage two holes are made through the leather to permit the legs to hang. When the rat is in the cage the split on the "back" of the cage is held closed by means of clips. When the rat has been in the cage for a minute or so, it usually relaxes and remains quite still (see Fig. 1).

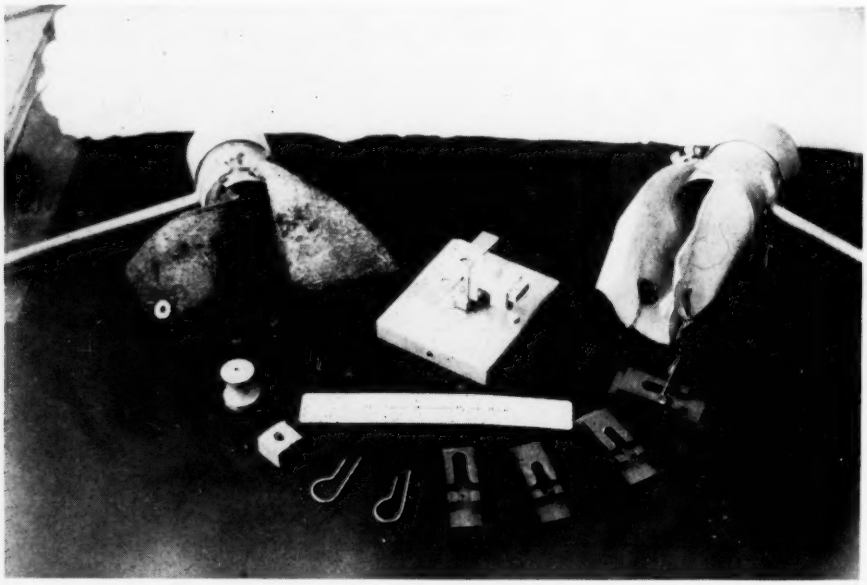

Fig. 1.—Photograph of rat holder and various types of rat foot holders.

*The Sphygmometer Cuff.*—The type of sphygmometer cuff necessary required standardization in making them. A study has been made of different cuffs varying in cross-sectional thickness of the wall and in cross-sectional diameter of the tube itself.

The cuffs are made as follows:

Natural Latex* is well emulsified and filtered through cheesecloth into a graduate. It is allowed to stand for one hour, after which time any minute air bubbles in the top of the emulsion are brushed off with scraps of filter paper.

Glass rods of the desired diameter, approximately ten to twelve inches long, are cleaned well, rinsed with distilled water, and then rinsed with 3 per cent ammonia water. They are allowed to air-dry and then are dipped into the latex. The rods are carefully (without touching the sides of the graduate) with-

*Obtained from General Latex and Chemical Co., Cambridge, Mass.

drawn at a constant rate of speed. (A geared electric motor with a spindle is very desirable.) The rate of withdrawal of the rod determines the wall uniformity and thickness of the rubber tube. Usually four inches a minute has been the satisfactory speed. A single dipping will give a thin rubber tube which does not stand excessive use. After permitting the rod to air-dry for 1.5 to 2 minutes, the rod is again immersed and withdrawn at the same speed. It is allowed to air-dry as before. Any minute air bubbles may be removed from the rod by blowing gently on the rod or by carefully touching the air bubbles with the torn edge of a piece of filter paper. The rod may be dipped as frequently as desired for building up the wall thickness.

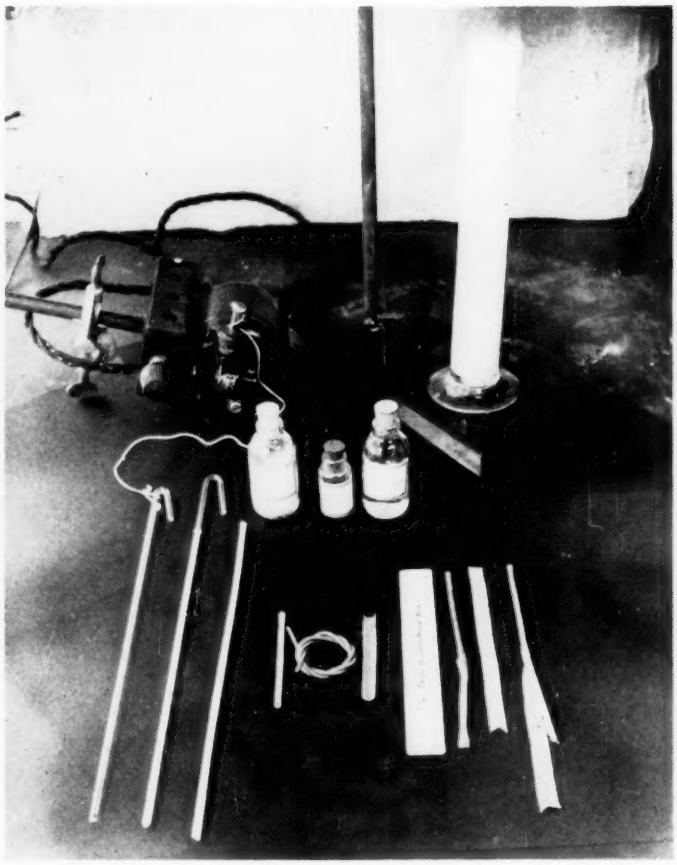

Fig. 2.—Preparation of sphygmometer cuffs.

The rod is then allowed to air-dry for seventy-two to ninety-six hours, or the process may be speeded up by heating in an oven at 99 to 100° C. for four to six hours. When dry, the rubber tubing is removed from the rod by lubricating thoroughly with a glycerine (3 Gm.)-talc (1 Gm.) mixture both externally and internally as it is freed from the rod. When completely free of the rod, the rubber tube is washed with water, dried, and lubricated with talc. Sections of desired length may be cut as needed.

The size cuff most practical and efficient has been found to be a 2-dip, five-inch per minute 5 mm. diameter tube. A length of approximately 7 cm. is cut off and sealed at one end with rubber cement. The other end is joined and cemented to 8 to 12 cm. of 5 mm. catheter tube. A 9 cm. piece of one-fourth or five-sixteenth inch ribbon is also cemented to the catheter by winding with a 5 mm. wide strip of latex cut from a sheet (see Fig. 2).

Cuffs of varying outside diameters and wall thicknesses have been prepared, and corresponding variations in the indicated blood pressure were determined. When cuffs less than 4 mm. diameter (when flattened out) are made by a 2-, or more, dip procedure, the wall thickness begins to play a role and excessively high recorded pressures ensue since the resistance of the rubber must be overcome in order to compress off the circulation into the foot. An initial standardization of cuffs, making a comparison with a direct puncture, would be advisable for one who has not had previous experience to be sure that the cuffs are of the proper thickness.

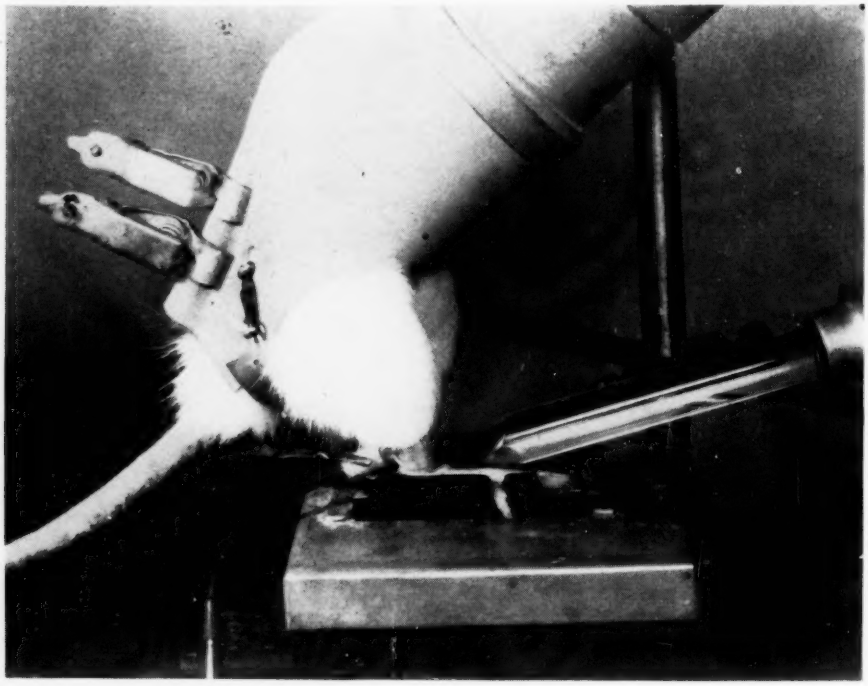

Fig. 3.—Illustration of positions of foot, foot holder, cuff, light, and position of rat for taking the blood pressure.

*The Photocell-Microammeter Volume Change Indicator.*—As shown in Fig. 3, the only light striking the photocell is that which passes through the foot from the light source. This value of light will remain constant as long as the foot is still and allowed to function normally. But when the flow of blood leaving the foot is restricted, the foot will begin to swell, causing a decrease in the amount of light striking the photocell. The circuit for the indicator is shown in Fig. 4. High impedance pentode tube acts as a load impedance for the high

impedance vacuum type photocell. The characteristic curves (Fig. 5) show that the potential of the common connection between the phototube and the pentode is determined by the intersection of their characteristics. It is also evident that a very small change of light on the photocell will result in a correspondingly large voltage change. This output voltage is applied to the grid

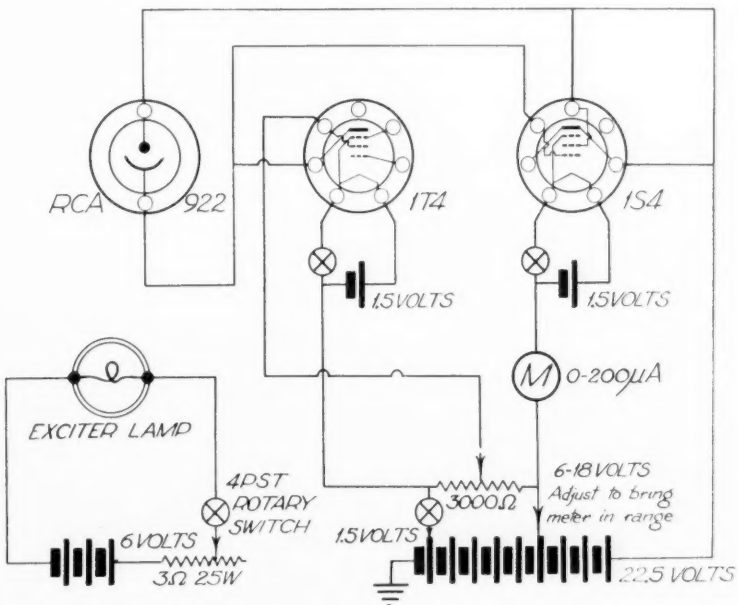


Fig. 4.—Circuit of the photocell microammeter volume change indicator.

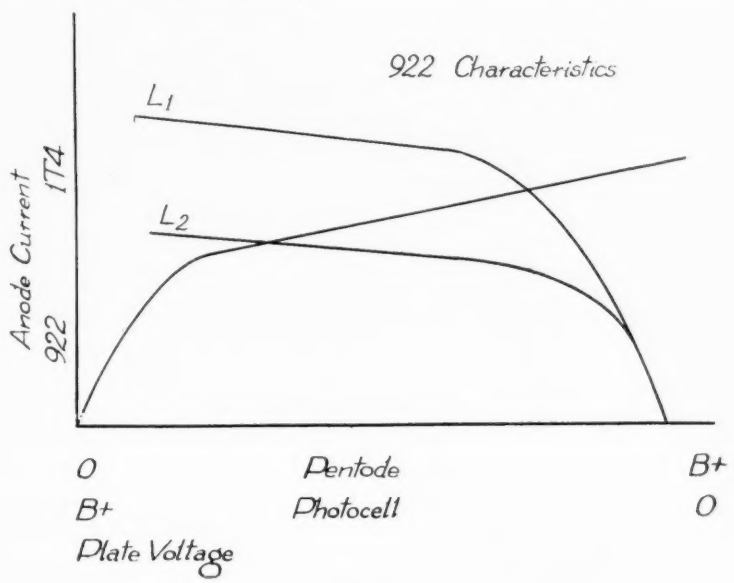


Fig. 5.

of the 1S4 output tube, the plate current of which is indicated on the 0 to 200 microammeter.[8]

*The Foot Holder.*—It must be pointed out that the footholder was designed not to hold the foot in position forcibly, but only to restrain if the rat attempts to withdraw the foot, so that the foot does not leave its correct position. This foot holder is not absolutely necessary but has been an aid in training the rats, and once trained they are conditioned to its feel. A hole directly under the place where the "palm" of the rat's foot rests is also made. This leads directly over the photocell. (See Figs. 1 and 3.)

*Sensitivity.*—By sensitivity is meant the responsiveness of the instrument to a given change in amount of light hitting the photocell. This is taken care of by the circuit itself.

*Accuracy.*—By accuracy is meant the amount of lag between the first change in volume in the foot caused by lowering the pressure of the sphygmometer cuff and the recording of the change in volume on the galvanometer. This accuracy is controlled by the intensity of the light source, the width of the cuff, and the size of the aperture, and also by the rate of lowering the sphygmometer cuff pressure. The intensity of light should be 10 to 35 foot-candles, when a Weston meter is held one inch from the source of light. The light should be directly over the aperture between one-half and one inch from the foot, so that the rays of light are perpendicular to the foot. The aperture in the foot holder which permits light through to the photocell can be from one-eighth to one-half inch in diameter. Interchangeable foot holders with holes of varying diameters have been used advantageously. The size of the cuff has been discussed.

*Source of Light.*—The light on the foot should be of a "heatless" type or transferred over the foot by a Lucite rod to eliminate heating the foot. A small 3.0 volt "lens type" "penlight" bulb has also been found convenient.

*Taking the Pressure.*—The rat is placed in the rat holder and allowed to remain for a minute or two with the two hind feet resting on the table or some other solid object. The cage should be clamped in a position so that the rat's body forms a 45- to 60-degree—$\measuredangle$—angle with the table (Fig. 3).

The cuff is then wound around the area just above the ankle and held there by wrapping the ribbon around the cuff and clamping it to the catheter tubing with a "light pressure" clamp so as not to constrict the catheter tubing. This cuff should be kept well powdered with talc. The cuff should be applied rather loosely (but not too loosely) since the venous pressure must not be exceeded or circulation will be stopped and no change in volume of the foot will occur and, therefore, there will be no reading. If no reading is obtained, the cuff should be rewound.

The rat's foot is placed in position in the foot holder and the extinguished light is brought directly over the back of the foot which lies directly over the hole in the foot holder. The instrument is turned on, and the circuit resistance control is adjusted so that the microammeter shows that the photocell is being activated. The cuff (attached ot the sphygmometer) is inflated to 250 mm., at which point the microammeter is adjusted to a reading of 150 microamperes by using a small rheostat on the light source. Small jumps or fluctuations on the

microammeter may be observed; these usually are due to movements of the foot of the rat (brought about by breathing, if the cage is not placed correctly, or by squirming) or to the interference of shadows falling on light-leaking parts of the photocell chamber. If less than ten foot-candles of light are used as the light source, shadows or reflections from the room lights onto the rat's foot will also cause erratic jumps of the microammeter needle. All extrinsic lights can be avoided by using a box over the instrument or by working in a darkened room.

The pressure is slowly lowered in the cuff at approximately 250 mm. to 0 mm. in 0.5 minute. When the systolic pressure is reached, a characteristic drop of the microammeter will be obtained. This drop will stop if the cuff is immediately inflated past the systolic pressure and will start again when the pressure in the cuff is again lowered. The microammeter will continue to approach zero

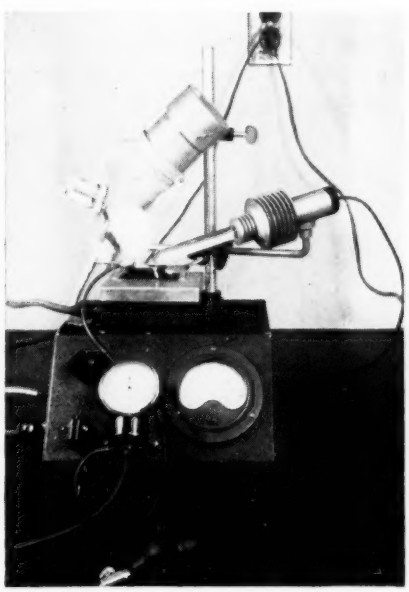

Fig. 6.—Assembled blood pressure instrument.

and may even speed up its dropping rate as pressure drops. The fall in microammeter reading is, of course, due to the change in volume of the foot incurred by blood flowing into the foot. This reduces the amount of light into the photocell.

When the pressure in the cuff gets below the venous pressure, the foot will recede to its original volume since blood can now leave the foot, and therefore the microammeter will rise to its higher reading. We have arbitrarily called this lower reading the "indicated" venous pressure, although its relationship to the actual venous pressure has not yet been established. The rate at which the cuff pressure is lowered, if too rapid, will give lower readings than if dropped more slowly, as is the case in any indirect blood pressure measurement. It has been our practice to get a range reading on the first trial by lowering the cuff

pressure at 250 mm. to 0 mm. in approximately 0.25 minute, followed by two or three readings at the standard rate of 0.5 minute for 250 millimeters. This, of course, is optional.

It has been observed that a waiting period of about one-fourth minute is necessary between readings. If no waiting period is taken, readings may not be obtained. See Fig. 6 for assembled blood pressure instrument.

## EXPERIMENTAL

All rats received from Rockland Farms, Carworth, or our own breeding colony are placed in individual cages and numbered. In most cases no training of rats was necessary, but occasional rats were very difficult to control. As an example of the variation of normal blood pressure in these normal animals, obtained by use of the described procedures, the blood pressure of a group of seventeen rats was determined for three consecutive days. The rats varied in weight from 190 to 320 grams. The sphygmometer cuff used was 5 mm. wide and 8 cm. long, two-dip, five-inch a minute latex tubing.

The average mean determination in this first group of animals was 118 ± 12 mm. for the first day, 116 ± 9 mm. for the second day, and 117 ± 8 mm. for the third day. (It has also been possible to take the blood pressure of rats as small as 50 grams in weight, with the appropriate foot holder and rat holder.)

## SUMMARY

A new method and instrument for the indirect determination of blood pressure of the rat has been described. This procedure does not require the heating or the anesthetization of the animal. The principle of its functioning is based on measuring the volume change in the foot by means of a photoelectric cell before and after application of pressure by means of a miniature sphygmometer cuff applied to the ankle of the foot.

By this method the average indicated systolic blood pressure of a group of seventeen normal, unheated, unanesthetized rats was found to be 117 mm. ± 8 mm. Hg. The indicated venous pressure was found to be 18 mm. ± 4 mm. Hg.

The authors are grateful to Miss L. Lipchuck, for assistances rendered in carrying out this work, and to Mr. L. McWilliams, for the photographs.

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# COMPARISON OF BLOOD PRESSURE MEASUREMENTS IN THE RAT AS OBTAINED BY USE OF THE TAIL AND FOOT METHODS AND BY DIRECT FEMORAL PUNCTURE

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A NEW method for the indirect determination of the blood pressure of the rat has been described.[1] This procedure has obviated the necessity of prewarming or anesthetizing the animal, as compared with the previously described methods in the literature.[2-7] It became of extreme interest to compare the blood pressures obtained by this method with the various methods under similar environmental conditions.

## EXPERIMENTAL

*The Tail Instrument.*—All determinations of blood pressure made on the tail were carried out using the same type of photocell-microammeter system as described for the foot.[1] The change in volume of the tail was measured by the decrease or increase of light entering the photocell from a slit formed between the upper surface of the tail and a fixed metal shield before and after constriction of the tail with a sphygmometer cuff (Fig. 1). (In measuring the volume change of the foot, the amount of light going through the foot was measured before and after constriction of the ankle with a sphygmometer cuff.) This instrument gave sensitivities to volume changes on the order of, or greater than, the instrument of Williams and co-workers[4] as modified by Shuler and associates[5] or Proskauer and associates.[6] Its sensitivity was also of the same order as the machine used on the foot throughout all the described experiments. The size of the cuff used on the tail was 9 mm. wide by 9 cm. long, dipped in the same manner as the sphygmometer cuff used on the foot and, therefore, of the same thickness of rubber. The cuff was wound around the base of the tail and held there by wrapping a ribbon 15 mm. wide around the cuff. The indirect blood pressure was taken in exactly the same manner as described for the Williams method except that the change in volume in the tail before and after constriction with the sphygmometer cuff was observed by the increase or decrease in light entering a photocell system rather than by a water plethysmograph.

*The Foot Instrument.*—The foot instrument has been described previously.[1]

*The Direct Puncture Manometer.*—The instrument used for the direct measurement of blood pressure was a modification of Hamilton's optical manometer.[8] Instead of using the diaphragm type of mechanism, our mirror was mounted on the end of a copper-beryllium Bourdon tube. The cross-sectional diameter of the tube used was approximately three-eighths inch, while its circumferential diameter was one and one-half inches.* The volume liquid displacement for the complete system (needle to copper tube, one-eighth inch O.D., to pet cock to Bourdon tube), between 0 and 300 mm. Hg pressure, was 0.1 cubic centimeter. By this system a linear relationship between pressure and mirror movement could be maintained from 0 to 300 mm. Hg pressure. Although the sensitivity of this method was much lower than that obtained by Hamilton's manometer, accuracy was maintained even at the higher blood pressure levels. The indicated pulse pressures of normal nembutalized rats were usually of the order of 5 to 15 mm. when pressures were taken from the femoral artery using a 23-gauge needle as a cannula.

From Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y.
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*United States Gauge, New York, N. Y.

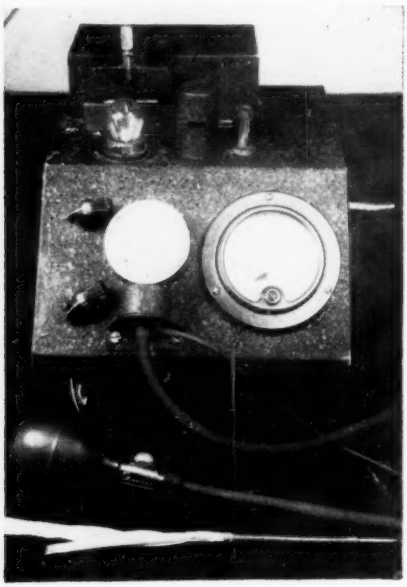

Fig. 1.—Volume change indicator adapted for tail.

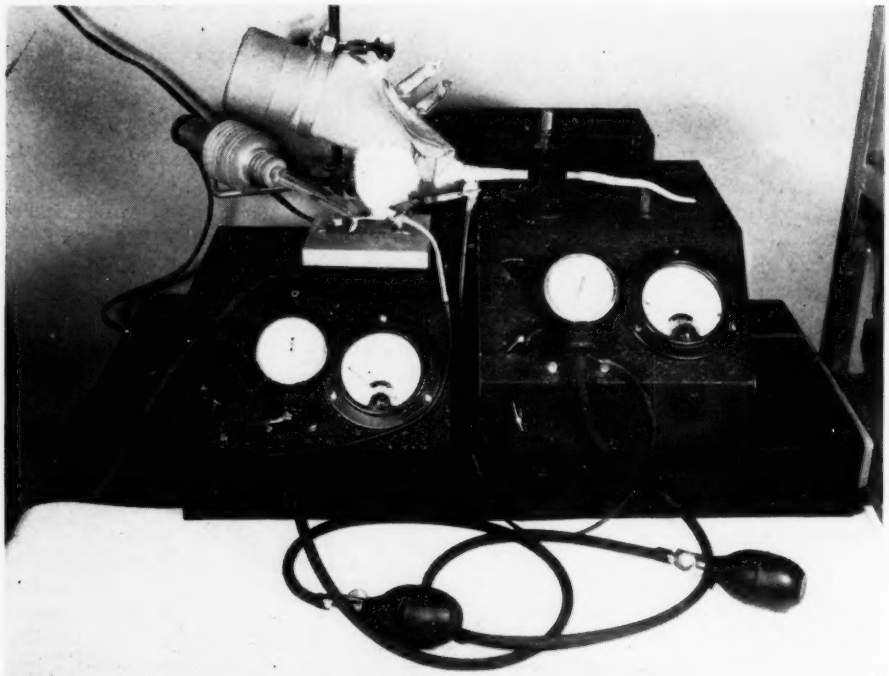

Fig. 2.—Tail and foot volume change indicators arranged for simultaneous measurement of blood pressure.

## RESULTS

*Deviations of Observed Readings in Normal Rats on the Same Day and on Different Days Under Normal and Heated Conditions by Use of Tail Method.*—Six rats were trained and the blood pressure was measured from the tail without warming the animal. Immediately after attempts were made to determine the blood pressure, the rat was placed in a carton container heated to the appropriate temperature by electric lamps. At the desired time it was removed from the carton box, and the blood pressure was again measured. The animal, as indicated in Table I, was allowed to remain at room temperature for the desired time, the blood pressure was determined, and the rat was inserted into the next carton box of desired temperature. The readings obtained are charted in Table I.

These results indicate:

1. The general unresponsiveness (no apparent volume change in the tail) of normal unanesthetized rats unless heated.

TABLE I. DEVIATION OF BLOOD PRESSURE IN TAIL OF NORMAL ANIMALS ON SAME DAY AND ON DIFFERENT DAYS UNDER NORMAL AND HEATED CONDITIONS

| RAT | DATE | ROOM TEMP.; NO HEAT | 42° 3 MIN. | ALLOWED TO COOL AT ROOM TEMP. | 42° 5 MIN. | ROOM TEMP. | 45° 5 MIN. | ROOM TEMP. | 47° 3 MIN. | ROOM TEMP. | 50° 3 MIN. | ROOM TEMP. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 19 | 6/13 | 0 | – | – | – | – | – | – | – | – | 145/45 | 80/35 |
| | 6/14 | 0 | 125/30 | 110/25 | – | – | – | – | 150/50 | 110/25 | 152/50 | 120/50 |
| | 6/18 | 0 | 95/20 * | 0 | 115/30 | 100/30 | 120/30 | 80/30 | – | – | – | – |
| | 6/19 | 0 | 85/20 | 0 | – | – | 115/30 | 60/30 * | – | – | 120/50 | 85/20 |
| 22 | 6/13 | 0 | – | – | – | – | – | – | – | – | 145/50 | 110/25 |
| | 6/14 | 0 | 0 | 0 | – | – | – | – | 110/30 * | 0 | 90/50 | 90/30 |
| | 6/18 | 0 | 0 | 0 | 95/30 | 60/30 | 90/30 | 70/30 | 140/30 | 80/25 | – | – |
| | 6/19 | 0 | 60/15 * | 0 | – | – | 70/20 | 60/20 | – | – | 70/50 | 60/20 |
| 25 | 6/13 | 0 | – | – | – | – | – | – | – | – | 130/50 | 0 |
| | 6/14 | 0 | 140/45 | | | – | – | – | 145/40 | 118/20 | 135/50 | 130/35 |
| | 6/18 | 0 | 110/40 | 0 | 125/60 | 76/40 | 125/45 | 70/30 | 120/30 | 70/22 | – | – |
| | 6/19 | 0 | 50/20 | 0 | – | – | 98/45 | – | – | – | 135/60 | 95/25 |
| 27 | 6/13 | 0 | – | – | – | – | – | – | – | – | 110/50 | 0 |
| | 6/14 | 0 | – | – | – | – | – | – | – | – | – | – |
| | 6/18 | 0 | 110/40 | 0 | 100/25 | – | 100/40 | 0 | – | – | – | – |
| | 6/19 | 0 | 0 | – | 0 | – | 60/30 ? | 0 | – | – | – | – |
| 28 | 6/13 | 0 | – | – | | | | | | | 100/40 | 0 |
| | 6/14 | 0 | – | – | – | – | – | – | – | – | – | – |
| | 6/18 | 0 | 60/20 * | 0 | 130/25 | 0 | 100/30 | 0 | – | – | – | – |
| | 6/19 | 0 | 0 | – | – | – | 125/30 | 60/20 * | | | | |
| 29 | 6/13 | 0 | – | | | | | | | | 110/50 | 80/20 * |
| | 6/14 | – | – | – | – | – | – | – | – | – | – | – |
| | 6/18 | 0 | 122/44 | 0 | 130/25 | 0 | 100/35 | – | – | – | – | – |
| | 6/19 | 0 | 120/35 | 0 | 110/40 | 0 | – | – | – | – | – | – |

0, No reading.
*, Poor reading (poor swing) due to small volume change.
–, Not done.

TABLE II. COMPARISON OF SIMULTANEOUSLY OBSERVED BLOOD PRESSURE BY USE OF TAIL AND FOOT METHOD IN UNHEATED, HEATED AND COOLED, NORMAL AND OPERATED ANIMALS

| | A TRAINED NORMAL RAT 50 | | | B TRAINED OPERATED RAT 68 | | | C TRAINED OPERATED RAT 29 | | | D HYPERTENSIVE RAT 34 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| TIME | FOOT | TAIL | | FOOT | TAIL | | FOOT | TAIL | | FOOT | TAIL | | |
| 0 | 110/20 | 0 | | 130/20 | 0 | | 130/25 | 110/30 | | 170/40 | 145/25 | | |
| 2 | 110/20 | 0 | Heat applied ← | 130/20 | 0 | Heat applied ← | 130/25 | 120/30 | Heat applied ← | 170/40 | 145/25 | Heat applied ← | |
| 4 | 110/20 | 0 | | 125/20 | 0 | | 160/50 | 145/40 | | 175/40 | 150/35 | (very gradual) | |
| 6 | 110/20 | 0 | | – | – | | 190/80 | 160/50 | | | | | |
| 8 | 115/20 | 75/? | | 135/50 | 130/50 | | 185/80 | 175/60 | | | | | |
| 10 | 130/30 | 95/? | | – | – | | 185/80 | 180/60 | Heat removed → | | | | |
| 12 | 140/60 | 115/35 | | – | – | | – | – | | 165/40 | 170/20 | | |
| 14 | 150/60 | 135/40 | | 145/50 | 140/50 | | 170/70 | 170/70 | | 170/35 | 170/35 | | |
| 16 | 145/60 | 135/40 | Heat removed → | – | – | | – | – | | – | – | | |
| 18 | 140/55 | 135/40 | | – | – | | – | – | Cage cooled ← | – | – | | |
| 20 | 125/60 | 130/50 | | 145/50 | 160/60 | Heat removed → | 160/90 | 150/45 | | 170/35 | 170/35 | | |
| 22 | 120/30 | 110/40 | Cage cooled ← | 145/50 | 160/50 | Cage cooled ← | 160/90 | 150/45 | | – | – | | |
| 24 | – | – | | 130/40 | 150/55 | | – | – | | – | – | | |
| 26 | 110/25 | 65/? | | 125/30 | 120/20 | | 145/60 | 130/35 | | – | – | | |
| 28 | 110/20 | ? | | – | – | | – | – | | 180/35 | 180/35 | | |
| 30 | 110/20 | ? | | 125/30 | 120/20 | | 145/80 | 120/30 | | | | | |
| 32 | 110/20 | 0 | | 125/30 | 90/? | | – | – | | 195/90 | 180/35 | Heat removed → | |
| 34 | – | – | | – | – | | 135/20 | 115/30 | | | | | |
| 36 | 110/20 | 0 | | 125/30 | 60/? | | – | – | | 205/90 | 205/90 | Cage cooled ← | |
| 38 | | | | – | – | | – | – | | 205/90 | 205/90 | | |
| 40 | | | | – | – | | 135/20 | 115/30 | | – | – | | |
| 42 | | | | 125/30 | 0? | | 135/20 | 110/30 | | 175/70 | 165/35 | | |
| 44 | | | | | | | 135/20 | 110/30 | | – | – | | |
| | | | | | | | | | | 170/40 | 145/40 | | 54 min. |
| | | | | | | | | | | 170/40 | 145/40 | | 58 min. |

0, No readings obtainable.
–, Not done.

2. The wide variation of "normal" blood pressure of normal animals when heated in the same way on different days.

3. The wide variation of response to given temperature changes of the same rat on different days.

*Comparison of Simultaneously Observed Blood Pressures by Use of Tail and Foot Methods in Unheated, Heated and Cooled, Normal and Operated Animals.*—Each animal used was placed in the rat holder adapted for foot blood pressure determination so that readings could be made simultaneously on the tail and on the foot (see Fig. 2). When warmed, the animals were heated by means of a portable heat lamp, so adjusted as not to reflect or shine directly into the apertures of the photocells of either machine.

The cuffs used in the tail method and the foot method were of such a diameter that they were proportional to the size of the appropriate appendage, that is, for the tail cuff, a 9 mm. wide, 9 cm. long cuff was used, while on the foot, where the cross-sectional diameter is smaller, a 5 mm. wide 7 cm. long cuff was used. Both cuffs were of the same thickness rubber drawn in the same speed from the same Latex (two-dip, 5 inches a minute).

In all cases, readings recorded were the average either of two or three readings. Sensitivities of both machines to volume change were the same. The results obtained in four rats picked at random are given in Table II.

These four experiments, which are a few of many such trials, indicate the following:

1. Not all rats gave volume changes in the tail when normal (unheated). In *all* cases (approximately 10,000 readings) blood pressure recordings (volume changes) were and have been obtained in using the foot method without heating the animal.

2. When warmed, those animals which gave no detectable volume change in the tail gradually gave increasingly larger volume changes in the tail at higher and higher blood pressure levels. When cooled by packing the metal portion of the cage with a cold water bag or ice, readings and volume changes gradually diminished until both were lost.

3. Readings were obtained continuously by use of the foot method with a definite rise of blood pressure due to heating. An increase of volume change was also observed. Heated rats are therefore no longer "normal" animals in reference to blood pressure.

4. The indicated blood pressure in the tail is not necessarily related to the indicated blood pressure in the foot in unheated unanesthetized animals, although in the heated animals the indicated blood pressures of both methods agree within the experimental error of the methods.

*Comparison of Observed Readings by Direct Femoral Puncture and by Tail and Foot Methods in Nembutalized Animals, and the Effect of Nembutal on "Vasoconstrictor Reflex."*—Three previously trained animals were used in this experiment. Before anesthetization, the blood pressure was measured both by the tail procedure and by the foot procedure simultaneously. The animal was then anesthetized intraperitoneally with nembutal, 5 mg. per 100 grams, and the blood pressure was followed as the anesthetic took effect. After anesthesia

TABLE III. CORRELATION OF FOOT METHOD AND TAIL METHOD BEFORE ANESTHESIA, AND CORRELATION OF BOTH METHODS WITH DIRECT PUNCTURE UNDER ANESTHESIA

| NORMAL RAT 17 | | | | | NORMAL RAT 19 | | | | | TRAINED OPERATED RAT 71 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| TIME (MIN.) | DIRECT | FOOT | TAIL | | TIME (MIN.) | DIRECT | FOOT | TAIL | | TIME (MIN.) | DIRECT | FOOT | TAIL | |
| 0 | – | 90/20 | 0 | Unanesthetized | 0 | – | 125/15 | 80/20 | Unanesthetized | 0 | | 110/30 | 0 | Unanesthetized |
| 3 | – | 90/20 | 0 → | Nembutalized | 2 | – | 125/20 | 80/20 → | Nembutalized | 5 | – | 110/30 | 0 → | Nembutalized |
| 9 | – | 95/20 | 50/20? | | 8 | | 155/40 | 125/20 | | 42 | | 130/25 | 95/20? | |
| 15 | – | 115/30 | 75/30 | | 17 | | 125/25 | 90/20 | | 44 | | 135/25 | 95/20 | |
| 26 | 90/ | 85/20 | 80/15 | | 27 | 120 | 125/20 | 110/20 | | 45 | | 140/25 | 95/25+ | |
| 30 | 90/ | 95/20 | 85/20 | | 42 | 120 | 125/20 | 110/20 | | 54 | 140 | 145/20 | 100/25 | |
| | | | | | | | | | | 57 | 140 | 145/20 | 100/25 ← | 0.4 c.c. hypertensin |
| | | | | | | | | | | 59 | 180 | 175/35 | 140/30 | |
| | | | | | | | | | | 59½ | 185 | 190/40 | 160/40 | |
| | | | | | | | | | | 59½ | 185 | 195/35 | 195/40 | |
| | | | | | | | | | | 59¾ | 185 | 185/40 | 185/50 | |
| | | | | | | | | | | 60 | 140 | 135/30 | 160/50 | |
| | | | | | | | | | | 61 | 125 | 125/30 | 130/25 | |
| | | | | | | | | | | 61½ | 130 | 125/30 | 115/20 | |
| | | | | | | | | | | 62 | 125 | 125/30 | 110/25 | |

0, No readings obtainable.
–, Not done.

was complete (third stage), the blood pressures were taken and the direct puncture instrument was then cannulated to the femoral artery on the right leg. The left foot was used to follow the blood pressure by the foot technique, while simultaneously observing the blood pressure in the tail.

In these three animals, obstruction of the upper respiratory passages due to the anesthetic used[7] was not encountered, and tracheotomy was not necessary.

In these three animals, the effect of anesthesia on the vasoconstrictor reflex is demonstrated (Table III). In the third animal various substances (pressor or depressor) were injected into the femoral vein and the blood pressure was followed by all three methods.

The results of these experiments indicate:

1. Normal unheated unanesthetized rats which gave no volume changes in the tail (and, therefore, presumably no blood pressure) when anesthetized with nembutal gradually gave volume changes in the tail as the degree of anesthesia increased without application of heat to the animal. It is, therefore, concluded that both anesthetization and heating of the animal have the same effect on the "vasoconstrictor reflex" in regard to the volume change and the indicated blood pressure in the tail. It therefore becomes apparent why agreement between the tail method and the direct puncture method is obtained in the anesthetized animal,[5, 7] but it does not necessarily mean that there is agreement between the indicated blood pressure using the tail method and the systemic blood pressure in the unanesthetized animal.

2. Agreement of indicated blood pressures obtained by the foot method, tail method, and direct puncture method in the anesthetized animal was within the limits of the experimental errors.

## SUMMARY

1. A comparison of indicated blood pressures as obtained by use of the foot and the tail has been made in both normal unheated and operated unheated animals. The agreement of the values obtained has been very poor, although both methods depend on the same principle for determination of blood pressure; that is, the change in volume in the appropriate appendage is observed by means of a photocell system before and after constriction of a sphygmometer cuff applied to the specific appendage.

2. The blood pressure of normal unheated rats as measured by the tail method has varied between 50 and 130 mm. where a volume change in the tail occurred. (Proskauer and co-workers[6] have reported a value of 65 to 95 mm. of Hg.) In very normal unheated animals, no volume change and, therefore, presumably no blood pressure could be detected. In all cases volume changes were observed when using the foot. The average blood pressure obtained in a group of seventeen normal unheated rats picked at random was 117 ± 8 mm. Hg.[1] This value is in good agreement with those obtained by direct puncture as reported by Schroeder[7] for normal anesthetized rats.

3. It was observed that in the tail of the normal unanesthetized animal, in cases where there was no volume change unless heated, the volume change gradually increased with increased heat applied to the rat, and that these

volume changes occurred at concurrently higher blood pressure levels. Attempts to standardize the amount and the rate of heating applied to the rat were made with no success. By using the foot method, readings were obtained without the application of heat. In the foot, as heat was applied, the volume change also increased but at a much lower rate, while the blood pressure rose from the level obtained in the unheated normal animal. It is, therefore, apparent that normal heated animals are no longer "normal" in regard to blood pressure. It is also apparent that the blood pressure in the tail, as indicated by the volume change principle, is not directly related to the systemic blood pressure in the unheated unanesthetized rat, although under abnormal conditions (heating or anesthesia) a relationship between indicated foot blood pressures and the indicated tail blood pressures was obtained.

4. Under the influence of anesthesia volume changes in the tail could be easily obtained without the application of heat, and agreement of all three methods was fairly good when simultaneous blood pressure readings were taken by tail, foot, and direct puncture.

The authors are indebted to Mr. Robert C. Annen and to Mr. Clifford Blauvelt for their contribution to the adaptation of the direct puncture instrument. We are also grateful to Mr. Leslie McWilliam for the photographs.

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# THE DIAGNOSIS OF PANCREATIC DISEASE BY ENZYME TESTS

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THE clinician has long found the diagnosis and treatment of diseases of the pancreas to be among the most difficult and uncertain of all diseases. This has been adjudged partly to be due to (1) the lack or obscurity of symptoms in early or moderately advanced stages of pancreatic disease clinically, and (2) the complexity of pancreatic tests and their impracticability for the practicing physician, biochemically.

In this paper I report the results of studies carried out from a series of patients seen in gastroenterologic practice which have been used as an aid in the diagnosis and treatment of diseases of the pancreas. This study was undertaken with the view of utilizing some procedure which would aid in establishing a diagnosis of pancreatic disease.

The American Gastroenterological Association[1] appointed a committee to study the pancreatic problem. Among other reports, two extensive investigations for the committee were published by two groups of investigators, one from the laboratories of the Mayo Clinic,[2] and one from the laboratories of the Western Reserve University School of Medicine and Hospital.[3]

I therefore believed it might be of interest to describe additional pancreatic studies in gastrointestinal diseases in the attempt to explore further the practicability and reliability of pancreatic diagnostic procedures.

These studies do not touch upon acute pancreatitis, which is in the nature of an acute surgical emergency, but rather on diseases of the pancreas such as cancer, stone, and chronic inflammation, as well as on disturbances of the pancreas caused by primary diseases of contiguous organs.

Extensive evidence has now been accumulated by surgeons and pathologists alike[4] that dyspepsia is often the primary or secondary expression of chronic pancreatitis. For example, Rich and Duff found "repeated focal necrosis, focal hemorrhages, and epithelial metaplasia" in routine pancreatic examinations during autopsies at the Johns Hopkins Hospital.[5] These findings have been duplicated by other observers.[6] Osler also drew attention to this fact and stated that the confusion existing on the subject was due to the "personal equation of the laboratory workers."[7]

Clinicians attempting an analysis of the role of chronic pancreatitis in causing dyspepsia are represented by Koehler,[8] who found that 26 per cent of his series of patients suffering from "functional indigestions" showed duodenal amylase deficiency. Rivers'[9] survey suggests a similar conclusion.

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Although pancreatic stones are rarely diagnosed by the clinician during life, the studies of Ludin[10] have shown that routine x-ray examinations of 542 pancreases correlated with histologic study revealed concretions in 5 per cent of the cases. These concretions varied in size from the head of a pin to that of a hazelnut. Snell and Comfort,[11] Moss and Freis,[12] and others have shown the value of pancreatic functional tests in patients with pancreatic lithiasis.

In reference to the condition described as "pancreatic edema," many surgeons have described the frequency of this condition at operation.[13] Archibald in 1913 was one of the first surgeons to report a series of patients suffering from attacks of acute epigastric pain in whom operation revealed only pancreatic edema.[14] Pratt,[15] a lifelong worker in pancreatic diseases, described the accumulative evidence which indicates that attacks of epigastric pain are due, not infrequently, to mild acute pancreatic lesions.

Experienced surgeons like Elman,[16] Cole,[13b] Demel,[17] Loeffler,[18] and others too numerous to mention all have reported series of patients suffering from attacks of epigastric pain in whom careful surgical exploration revealed diffuse edema of the pancreas, without evidence of necrosis or hemorrhage.

Pratt[15] has emphasized the fact that in patients where the symptoms are indicative of gall bladder disease, or where the clinical picture is that of gallstones and the cholecystograms are negative, pancreatic disease is frequently present.

The need for study of pancreatic disorders has thus been keenly felt for some time.

Since Einhorn's introduction of the duodenal bucket in 1910[20] numerous investigators[21-26] have reported the results of their procedures in the testing of pancreatic function. However, the recent studies of Free and Myers[26] present such a comprehensive and improved system of technique that I have used their methods in testing pancreatic function.

## METHOD

A series of eight subjects in good health who were free of any gastrointestinal signs or symptoms were selected for normal controls. They consisted of five women and three men ranging from 20 to 58 years of age. Duodenal drainages and tests were routinely performed in all subjects on an empty stomach in the morning. The fasting duodenal secretion was first extracted and then one ounce of warm olive oil was injected through the tube into the duodenum and retained for ten minutes. The duodenal contents were siphoned off in twenty-, forty-, and sixty-minute periods after the olive oil stimulation and each specimen was examined for proteinase, amylase, and lipase according to the methods devised by Free and Myers.[26] Thus twelve pancreatic functional determinations were performed at each duodenal drainage. As described in Tables I to VI, the number of duodenal drainages varied in each patient from one to five, giving a range of functional tests numbering from twelve to sixty for each subject. In all, a total of more than one thousand duodenal pancreatic tests are herein reported. Bile pigment in each diluted sample was determined by the icterus index.

## CASE MATERIAL AND RESULTS

Table I shows that in over 288 pancreatic enzyme determinations taken from eight normal subjects, the average values for the fasting and twenty-, forty-, and sixty-minute periods of stimulation by olive oil were: proteinase,

3.6 mg.; amylase, 7.5 mg.; and lipase, 8 milliliters. However, it is noted that the normal variations for proteinase, amylase, and lipase are great. They range from 0.6 to 7.4 mg. in proteinase, from 0 to 17.3 mg. in amylase, and from 0 to 31.4 ml. in lipase. In these normal subjects zero readings were not found again at subsequent tests.

TABLE I. DUODENAL CONTENT ANALYSES FOR PANCREATIC ENZYMES IN EIGHT NORMAL SUBJECTS OVER TWENTY-FOUR TEST PERIODS; AVERAGE VALUES, 288 TESTS

| ENZYME | FASTING | 20 MIN. AFTER OLIVE OIL STIMULATION | 40 MIN. AFTER OLIVE OIL STIMULATION | 60 MIN. AFTER OLIVE OIL STIMULATION | MINIMUM VARIATION | MAXIMUM VARIATION |
|---|---|---|---|---|---|---|
| Proteinase* | 2.5 | 3.0 | 4.2 | 4.9 | 0.6 | 7.4 |
| Amylase† | 5.2 | 6.4 | 8.2 | 10.1 | 0 | 17.3 |
| Lipase‡ | 4.8 | 5.7 | 6.8 | 15.0 | 0 | 31.4 |

*Milligrams tyrosine equivalent.
†Milligrams glucose equivalent.
‡Milliliters 0.05 N sodium hydroxide equivalent.

Table II shows that in over sixteen pancreatic enzyme determinations taken from eight subjects with "functional" or nonorganic disturbances of the upper gastrointestinal tract, the average values for the fasting and twenty- and sixty-minute stimulation intervals were: proteinase, 2.0 mg.; amylase, 4.3 mg.; and lipase, 4.0 milliliters. The individual variations in "functional" disorders are considerable. They range from 0.2 to 3.8 mg. in proteinase, from 0.6 to 8.5 mg. in amylase, and from 0 to 8.5 ml. in lipase. Zero findings were not repeated at subsequent determinations. These eight subjects were diagnosed as having upper digestive tract dyspepsia, six from gastrointestinal neuroses and two from the postcholecystectomy syndrome.

TABLE II. DUODENAL CONTENT ANALYSES FOR PANCREATIC ENZYMES IN EIGHT PATIENTS WITH "FUNCTIONAL" OR NONORGANIC DISORDERS OF THE UPPER GASTROINTESTINAL TRACT; AVERAGE VALUES FOR SIXTEEN TESTING PERIODS, 192 TESTS

| ENZYME | FASTING | 20 MIN. AFTER OLIVE OIL STIMULATION | 40 MIN. AFTER OLIVE OIL STIMULATION | 60 MIN. AFTER OLIVE OIL STIMULATION | MINIMUM VARIATION | MAXIMUM VARIATION |
|---|---|---|---|---|---|---|
| Proteinase* | 1.3 | 1.8 | 2.2 | 2.7 | 0.2 | 3.8 |
| Amylase† | 2.9 | 4.0 | 4.9 | 5.6 | 0.6 | 8.5 |
| Lipase‡ | 2.9 | 3.4 | 4.5 | 5.1 | 0 | 8.5 |

*Milligrams tyrosine equivalent.
†Milligrams glucose equivalent.
‡Milliliters 0.05 N sodium hydroxide equivalent.

Table III shows that in twenty subjects suffering from organic diseases of the upper digestive tract, the average values for all the intervals previously described were: proteinase, 1.3 mg.; amylase, 3.7 mg.; and lipase, 3.6 milliliters. The individual test readings for enzymes here also are very variable. The range for proteinase is from 0 to 5.6 mg.; for amylase, from 0 to 10.4 mg.; and for lipase, from 0 to 13.6 milliliters. In contrast with the normal controls and the "functional" cases, if zero readings were obtained and found again at subsequent tests, extensive involvement of the pancreas by cancer was present as occurred in the three cases of cancer of the pancreas (Table V, Cases

TABLE III. DUODENAL CONTENT ANALYSES FOR PANCREATIC ENZYMES IN TWENTY PATIENTS WITH ORGANIC DISEASES OF THE UPPER GASTROINTESTINAL TRACT; AVERAGE VALUES FOR FORTY-THREE TESTING PERIODS, 510 TESTS

| ENZYME | FASTING | 20 MIN. AFTER OLIVE OIL STIMULATION | 40 MIN. AFTER OLIVE OIL STIMULATION | 60 MIN. AFTER OLIVE OIL STIMULATION | MINIMUM VARIATION | MAXIMUM VARIATION |
|---|---|---|---|---|---|---|
| Proteinase* | 0.7 | 1.2 | 1.5 | 1.7 | 0 | 5.6 |
| Amylase† | 2.2 | 3.0 | 3.4 | 6.1 | 0 | 10.4 |
| Lipase‡ | 0.9 | 3.0 | 4.8 | 5.7 | 0 | 13.6 |

*Mill.grams tyrosine equivalent.
†Milligrams glucose equivalent.
‡Milliliters 0.05 N sodium hydroxide equivalent.

3, 4, and 18). If near zero readings were consistently obtained, particularly in proteinase, extensive disease of the pancreas was found such as in the three cases of pancreatic cirrhosis with or without lithiasis (Table V, Cases 2, 6, and 7).

## COMMENT

The twenty patients with organic disease listed in Tables III and VI consisted of three patients with carcinoma of the pancreas, two with cholithiasis, one with choledocholithiasis, one with pancreatic lithiasis, two with pancreatic cirrhosis (diagnosed at operation), three with cirrhosis of the liver, three with duodenal ulcer, one with gastric ulcer, and one with infectious hepatitis and cholangitis, carcinoma of the pyloric end of the stomach, carcinoma of the gall bladder and bile ducts, and nontropical sprue, respectively.

Table IV compares the total average enzyme values for all interval readings found in normal subjects with those of functional and organic diseases of the upper digestive tract. It is seen that the respective average readings in the "normal" controls and the "functional" cases appear to be clearly defined. However, the difference between the average readings of the "functional" and those of the "organic" diseases seems small. This lack of contrast appears to be due to the inclusion of a group of cases under "organic" causes in which the pancreas itself was not primarily involved. Cases of gastric and duodenal ulcer or cholelithiasis are examples. On the other hand, when the cases in which the pancreas is involved primarily, such as by carcinoma or cirrhosis or the stone, are examined the test readings are consistently zero or very close to zero. The minimal and maximal ranges varied quite widely in any one patient in whom the pancreas was not extensively and markedly involved and rendered it impossible to determine accurately the category in which one reading placed a patient.

Percentages were not calculated since the range of variation in (1) normal subjects, (2) patients with organic disease of the upper digestive tract, and (3) patients with functional diseases of the upper digestive tract are so great that only repeated zero readings or repeated near zero readings are of diagnostic significance.

One single determination of the proteinase, amylase, or lipase values would appear to be adequate since the enzyme values were consistent in rendering the same information in one patient. This was particularly true where severe

TABLE IV. DUODENAL CONTENT ANALYSES FOR PANCREATIC ENZYMES; TOTAL AVERAGE OF FOUR ENZYME READINGS IN COMPARATIVE STUDIES OF UPPER GASTROINTESTINAL TRACT DISEASES

| ENZYME | NORMAL CONTROL SUBJECTS | FUNCTIONAL OR NONORGANIC DISEASES | ORGANIC DISEASES |
|---|---|---|---|
| Proteinase* | 3.6 | 2.0 | 1.3 |
| Minimum variation | 0.6 | 0.2 | 0 |
| Maximum variation | 7.4 | 3.8 | 5.6 |
| Amylase† | 7.5 | 4.3 | 3.7 |
| Minimum variation | 0 | 0.6 | 0 |
| Maximum variation | 17.3 | 8.5 | 10.4 |
| Lipase‡ | 8.0 | 4.0 | 3.6 |
| Minimum variation | 0 | 0 | 0 |
| Maximum variation | 31.4 | 8.5 | 13.6 |

*Milligrams tyrosine equivalent.
†Milligrams glucose equivalent.
‡Milliliters 0.05 N sodium hydroxide equivalent.

TABLE V. ENZYME VALUES IN EXTENSIVE ADVANCED PANCREATIC DISEASE PROVED AT OPERATION

| CASE | DIAGNOSIS | NUMBER OF TESTS | FASTING | | | 20-MIN. STIMULATION | | | 40-MIN. STIMULATION | | | 60-MIN. STIMULATION | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | P. | L. | A. | P. | L. | A. | P. | L. | A. | P. | L. | A. |
| 3 | Carcinoma of head of pancreas | 1 | 0 | 0 | 0 | | 0 | | | 0 | | | 0 | |
| | | 2 | 0 | 0 | 0 | | 0 | | | 0 | | | 0 | |
| 2 | Advanced pancreatic cirrhosis | 1 | 0 | 0 | 0 | 0.2 | 0.7 | 0.5 | 0.5 | 1.8 | 0.8 | 0.5 | 2.4 | 1.0 |
| | | 2 | 0 | 0 | 0 | 0.4 | 1.5 | 1.1 | 0.4 | 1.7 | 1.3 | 0.4 | 2.1 | 1.0 |
| | | 3 | 0.2 | 0.8 | 1.5 | 0.2 | 1.3 | 1.2 | 0.5 | 2.1 | 1.0 | 0.3 | 1.5 | 1.3 |
| | | 4 | 0 | 0 | 0 | 0 | 0.5 | 0.3 | 0.3 | 0.9 | 0.9 | 0.4 | 1.5 | 1.0 |
| | | 5 | 0.5 | 1.9 | 1.0 | 0.5 | 1.4 | 1.0 | 0.5 | 1.8 | 1.3 | 0.6 | 2.4 | 1.0 |
| 4 | Carcinoma of head and body of pancreas | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 7 | Advanced cirrhosis of the liver and pancreas | 1 | 0 | 0 | 0 | 0.2 | 0.8 | 0.7 | 0.3 | 1.8 | 0.8 | 0.2 | 3.2 | 1.0 |
| | | 2 | 0 | 0 | 0 | 0.2 | 1.5 | 0.3 | 0.2 | 1.9 | 0.8 | 0.1 | 2.6 | 1.1 |
| 18 | Carcinoma of head and body of pancreas | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 6 | Extensive pancreatic lithiasis with cirrhosis | 1 | 0 | 0.8 | 0.3 | 0.2 | 1.5 | 0.7 | 0.2 | 2.1 | 0.3 | 0.3 | 2.4 | 0.4 |

P., Proteinase; L., lipase; A., amylase.

TABLE VI. AVERAGE ENZYME VALUE IN ORGANIC DISEASES OF THE UPPER DIGESTIVE TRACT

| CASE | DIAGNOSIS | NUMBER OF TESTS | PROTEINASE | AMYLASE | LIPASE |
|---|---|---|---|---|---|
| 1 | Cholelithiasis, cholecystitis | 12 | 1.1 | 2.2 | 3.2 |
| 5 | Carcinoma, gall bladder | 4 | 0.9 | 2.6 | 3.3 |
| 8 | Cirrhosis of liver | 12 | 2.95 | 4.9 | 7.2 |
| 9 | Nontropical sprue | 4 | 3.1 | 7.1 | 8.6 |
| 10 | Choledocholithiasis | 8 | 1.2 | 4.5 | 4.5 |
| 11 | Cholecystitis with lithiasis | 8 | 1.3 | 6.5 | 3.9 |
| 12 | Penetrating duodenal ulcer | 8 | 1.2 | 3.0 | 4.5 |
| 13 | Chronic duodenal ulcer | 8 | 1.9 | 6.1 | 7.6 |
| 14 | Chronic duodenal ulcer | 8 | 1.5 | 4.2 | 4.2 |
| 15 | Gastric ulcer | 8 | 2.4 | 7.6 | 6.3 |
| 16 | Carcinoma, pyloric stomach | 8 | 1.2 | 2.6 | 2.5 |
| 17 | Cirrhosis of liver | 8 | 1.6 | 4.5 | 3.9 |
| 19 | Chronic cholecystitis | 8 | 1.7 | 2.9 | 2.7 |
| 20 | Infectious epidemic hepatitis | 8 | 1.7 | 4.2 | 4.4 |

primary disease of the pancreas was present, as in the three cases of carcinoma of the pancreas, each accompanied by jaundice.

In the three patients with pancreatic carcinoma the enzyme values were zero throughout for every proteinase, amylase, and lipase reading at the fasting and twenty-, forty-, and sixty-minute stimulation periods. In the case of marked pancreatic cirrhosis with or without lithiasis, the readings were all consistently close to zero. These two primary diseases of the pancreas, that is, cancer or extensive cirrhosis (with or without stone), therefore consistently gave findings of diagnostic value in the duodenal pancreatic enzyme tests.

## SUMMARY

1. Over 1,000 pancreatic enzyme tests (Free and Myers[26]) were made from the duodenal secretions in a group of normal subjects, in a group of patients with "functional," or nonorganic disease of the upper digestive tract, and in a group of patients with organic disease of the pancreas, biliary tract, stomach, or duodenum.

2. The range and mean of duodenal pancreatic enzyme tests for normal subjects and patients suffering from upper digestive tract disorders are described. Variations of test findings are very wide in both normal subjects and patients with most organic diseases of the pancreas or upper digestive tract.

3. Normal subjects as well as patients with extensive and severe pathologic involvement of the pancreas such as cancer may give zero or near zero test readings in individual duodenal pancreatic enzyme tests.

4. In patients with organic disease of the stomach or biliary tract, pancreatic duodenal enzyme tests revealed no individual distinctive or diagnostic findings. However, their group mean value was definitely lower than the comparable value in normal subjects.

5. A group of ten patients with chronic low-grade or moderate inflammation of the pancreas, as revealed at operation, had a series of duodenal pancreatic enzyme tests performed before and after operation. The duodenal enzyme study failed to show any consistently abnormal findings of diagnostic value either pre- or postoperatively.

6. In six cases of extensive involvement of the pancreas by cancer or cirrhosis with pancreatic lithiasis, 125 tests gave abnormal findings in the duodenal pancreatic enzyme tests. These abnormal findings were either zero or near zero values.

7. If a zero reading was found in the pancreatic enzyme tests of a normal subject, it was not consistently found at subsequent determinations. In the presence of advanced and extensive pancreatic disease such as cancer or cirrhosis, zero and near zero readings were found consistently at all subsequent determinations.

## CONCLUSION

Extensive and advanced disease of the pancreas such as cancer or cirrhosis with lithiasis gave repeated zero or near zero readings in the duodenal pan-

creatic enzyme tests. Pancreatic enzyme tests are useful in diagnosing advanced and extensive disease of the pancreas.

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# INTRAVENOUSLY ADMINISTERED GELATIN—A TOXICITY STUDY

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IT IS generally recognized that an infusion of blood or plasma is the most effective means of compensating for fluid loss which occurs in hemorrhage and shock of various types. Since blood and plasma are not always immediately at hand, however, it is desirable that a plasma substitute be found which is innocuous, effective, inexpensive, and readily available.

The recent literature records that substitutes somewhat less effective than plasma in restoring circulating fluid volume, but much more effective than crystalloidal solutions, are aqueous solutions of macromolecular substances, such as gelatin, gum arabic, pectin, ovalbumin, polyvinyl alcohol, and methyl cellulose. Gelatin[1-20] appears to possess desirable characteristics to a greater degree than most of the other macromolecular substances mentioned.

Observations in this laboratory on the toxic effects in animals of infused gelatin solutions which were subsequently tested for efficacy by laboratory animal experimentation[11, 18] and clinical investigation [15, 16, 19, 20] confirm the findings of others in many respects. Some of the undesirable properties of gelatin, however, such as impairment of the production of plasma proteins and hemoglobin,[17] and degenerative and proliferative arterial lesions of the sclerosing type reported by Heuper[6] in dogs, are apparently observed only when large amounts of highly concentrated gelatin solution are repeatedly infused into the blood stream of intact animals, thus placing a severe strain on the circulatory system. When a 5 to 8 per cent gelatin solution is infused into dogs or human subjects suffering fluid loss from hemorrhagic, traumatic, or burn shock in amounts approximately equal to the amount of fluid which has escaped, such untoward effects have not been reported.

The experiments herewith presented were designed to test whether or not the circulatory system of dogs under the stress of light anesthesia and severe blood loss could tolerate large quantities of 5 per cent gelatin administered in divided doses over a period of several weeks.

The solutions of gelatin* were prepared by electrodialysis of osseous calcium gelatinate. The 5 per cent solutions preserved with phenyl-mercuric-borate, 1:25,000, were degraded by autoclaving to obtain a mean molecular weight of 20,000 (number average). These solutions were nonantigenic and nonpyrogenic.

## EXPERIMENTAL

Nine mongrel dogs supplied with a high protein diet supplemented with raw liver were bled of 29 to 54 per cent of their estimated blood volume at weekly intervals for periods of two to thirteen weeks (average, seven weeks). The blood, aseptically removed from the

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*Supplied by Dr. S. H. Eppstein, of these laboratories.

exposed jugular veins under light ether anesthesia, was replaced at the same site by infusion of 5 per cent gelatin solution in amounts equal to 80 per cent of the blood removed. Control animals consisted of two dogs bled just as severely as those mentioned, but with no parenteral fluid replacement, and twenty apparently healthy dogs with no treatment whatsoever.

At intervals determinations of plasma protein and gelatin concentration in plasma, urine, and aqueous tissue extracts* were made by the method of Waters[4] which makes use of the fact that gelatin is not precipitated by trichloracetic acid but is precipitated by acid tungstate. Plasma protein nitrogen was thus determined by the usual Kjeldahl technique on the trichloracetic acid precipitate and gelatin nitrogen by difference between nitrogen contents of the acid tungstate and trichloracetic acid precipitates. The usual methods were employed for determination of red cell volume, hemoglobin, red cell count, white cell count, and sedimentation rate.[22]

Extensive pathologic studies on tissues of experimental and control dogs were done.†

On one of the nine dogs, a celiotomy was performed both prior to and one week after a series of six hemorrhages and gelatin infusions. Liver tissue was removed and examined at these times. Seven weeks later this dog was sacrificed and the tissues examined. A second dog was bled and infused with gelatin five times after a celiotomy had been performed for removel of liver tissue. He was sacrificed two weeks after the last hemorrhage and infusion.

## RESULTS

Repeated massive hemorrhages and infusions of purified gelatin in dogs under the conditions described produced no notable changes in deportment or body weight (Table I). The only gross disturbance observed was the formation of thrombi at the sites of injection in some dogs which Patek and co-workers[24] have demonstrated to be due to the mercurial preservative employed. Histologic examination of the jugular veins at autopsy revealed no changes in the intima. Considerable scar tissue formed about these veins, presumably due to the surgery involved, and fluid regurgitated into surrounding tissue when the needle was withdrawn. Wound healing was not impaired. There was no indication of the presence of pyrogenic or antigenic material in the gelatin.

TABLE I. SHOWING THE EFFECT OF REPEATED MASSIVE HEMORRHAGES AND GELATIN INFUSIONS ON THE BODY WEIGHTS OF DOGS

| DOG | NUMBER OF HEMORRHAGES AND INFUSIONS (WK.) | INITIAL BODY WEIGHT (KG.) | CHANGE IN BODY WEIGHT (KG.) | AMOUNT OF BLOOD REMOVED (ML.) | AMOUNT OF 5% GELATIN INFUSED (ML.) |
|---|---|---|---|---|---|
| 2 | 13 | 12.3 | +2.6 | 5,668 | 4,534 |
| 3 | 12 | 12.4 | +6.4 | 7,366 | 5,893 |
| 4 | 3 | 19.8 | +0.2 | 2,345 | None |
| 5 | 12 | 15.6 | +4.8 | 8,531 | None |
| 6 | 7 | 15.5 | +1.3 | 3,532 | 2,826 |
| 10 | 7 | 9.5 | +0.8 | 2,250 | 1,800 |
| 11 | 7 | 13.4 | +0.1 | 3,530 | 2,824 |
| 12 | 2 | 11.6 | +2.4 | 905 | 725 |
| 13 | 2 | 10.2 | +0.7 | 640 | 520 |
| 14 | 6 | 19.8 | −1.3 | 3,155 | 2,524 |
| 15 | 5 | 19.0 | −0.8 | 2,410 | 1,928 |

*Weighed quantities of kidney and liver from both control and gelatin-infused dogs were thoroughly macerated with saline in a Waring blendor. Gelatin in the supernatant fluid was determined by the method used for plasma gelatin.

†By Dr. Walter Schiller and Dr. Bruno Volk.

Examination of blood and plasma during the experimental period revealed that the changes observed in hemoglobin and plasma protein levels could be accounted for by the severe hemorrhage alone and were not markedly affected by the infused gelatin (Tables II and III).

TABLE II. HEMOGLOBIN, RED CELL VOLUME, AND PLASMA PROTEIN NITROGEN LEVELS OF DOGS SUBJECTED TO WEEKLY MASSIVE HEMORRHAGES BOTH WITH AND WITHOUT REPLACEMENT OF THE WITHDRAWN BLOOD BY GELATIN SOLUTION

| | TEST GROUP (WITHDRAWN BLOOD REPLACED BY GELATIN) | | | | | | | | | CONTROL GROUP (NO PARENTERAL FLUID REPLACEMENT) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | DOG 2 | | | DOG 3 | | | DOG 6 | | | DOG 4 | | | DOG 5 | | |
| TIME (WK.) | Hg. (GM. %) | CELL VOL. (%) | PPN (Mg. %) | Hg. (GM. %) | CELL VOL. (%) | PPN (Mg. %) | Hg. (GM. %) | CELL VOL. (%) | PPN (Mg. %) | Hg. (GM. %) | CELL VOL. (%) | PPN (Mg. %) | Hg. (GM. %) | CELL VOL. (%) | PPN (Mg. %) |
| 1 | | 48.1 | 1,010 | 14.0 | 45.6 | 843 | 16.5 | 48.9 | 978 | 20.8 | 58.8 | 924 | 11.6 | 36.4 | 811 |
| 2 | 13.2 | 44.0 | 930 | 8.9 | 31.8 | 825 | 18.5 | 55.0 | 1,025 | 13.6 | 44.4 | 890 | 8.9 | 31.3 | 865 |
| 3 | 12.2 | 40.8 | 977 | 11.0 | 41.4 | 840 | 13.9 | 44.1 | 1,022 | 11.0 | 36.6 | 838 | 9.5 | 32.1 | 728 |
| 4 | 11.2 | 39.6 | 1,006 | 11.4 | 40.8 | 735 | | 31.3 | 873 | | | | 9.9 | 36.3 | 903 |
| 5 | (No hemorrhage or infusion) | | | 14.8 | 46.8 | 886 | 10.4 | 37.5 | 914 | | | | 9.3 | 32.5 | 923 |
| 6 | 13.8 | 46.8 | 969 | 12.6 | 45.6 | 829 | 9.3 | 35.0 | | | | | | 27.5 | 777 |
| 7 | 11.6 | 40.8 | 879 | 14.0 | 46.8 | 861 | | 31.3 | 919 | | | | 9.9 | 35.0 | 855 |
| 8 | 11.0 | 40.8 | 904 | 13.4 | 45.0 | 847 | | | | | | | 10.1 | 37.5 | |
| 9 | 10.7 | 39.6 | 992 | 14.6 | 47.5 | 926 | | | | | | | | 30.0 | 817 |
| 10 | 11.0 | 40.8 | 904 | 13.4 | 43.8 | 840 | | | | | | | 9.4 | 35.0 | 876 |
| 11 | 9.5 | 35.4 | 908 | 11.6 | 40.6 | 797 | | | | | | | 9.1 | 33.5 | |
| 12 | 9.7 | 32.9 | 956 | 12.8 | 45.0 | 792 | | | | | | | 7.9 | 30.5 | 948 |
| 13 | 8.3 | 31.3 | 898 | | | | | | | | | | | | |
| 14 | 9.7 | 36.0 | 889 | | | | | | | | | | | | |

TABLE III. ILLUSTRATING TYPICAL RATES OF PLASMA PROTEIN REGENERATION AND DISAPPEARANCE OF PLASMA GELATIN IN DOGS (DATA FROM DOG 3 OBTAINED ON TENTH WEEK OF HEMORRHAGE AND INFUSION STUDIES; FOR FURTHER CONDITIONS OF EXPERIMENT, SEE TEXT)

| TIME (HR.) | PLASMA PROTEIN NITROGEN (MG. %) | PLASMA GELATIN NITROGEN (MG. %) | SEDIMENTATION RATE (MM/HR.) |
|---|---|---|---|
| Before hemorrhage | 840 | 0 | 1.0 |
| Blood withdrawn (658 ml. or 3.6% of body weight) and 520 ml. of 5% gelatin infused | | | |
| 1/4 | 326 | 281 | 70.0 |
| 2 | 435 | 178 | 56.0 |
| 4 | 482 | 139 | 50.0 |
| 7 | 541 | 110 | 37.0 |
| 11 | 566 | 90 | 30.0 |
| 16 | 621 | 65 | 27.0 |
| 22 | 701 | 35 | 28.0 |
| 30 | 730 | 32 | 27.0 |
| 48 | 774 | 18 | 43.0 |
| 168 | 797 | 12 | 1.0 |

The sedimentation rate of erythrocytes was greatly accelerated by the infused gelatin. In most instances the sedimentation rate decreased approximately at the same rate as the gelatin concentration in the plasma decreased. In all instances, the rate had returned to normal the following week (Table III).

Plasma gelatin determinations revealed the presence of approximately 80 per cent of that infused at one-fourth hour after infusion, 50 per cent at four hours, and 25 per cent at sixteen hours. Urine gelatin determinations revealed that only 37 to 62 per cent of the gelatin infused was excreted unchanged (Table IV). This occurred within twenty-four hours. Gelatin determinations on aqueous extracts of the liver and kidneys of one dog at autopsy eight days after thirteen infusions indicated that there was no free gelatin stored in these organs.

TABLE IV. RATE OF APPEARANCE OF GELATIN IN URINE FOR THREE SUCCESSIVE WEEKS (DATA FROM DOG 3)

| FIRST WEEK | | SECOND WEEK | | THIRD WEEK | |
|---|---|---|---|---|---|
| PERIOD AFTER INFUSION (HR.) | AMOUNT OF GELATIN IN URINE (% OF GELATIN INFUSED) | PERIOD AFTER INFUSION (HR.) | AMOUNT OF GELATIN IN URINE (% OF GELATIN INFUSED) | PERIOD AFTER INFUSION (HR.) | AMOUNT OF GELATIN IN URINE (% OF GELATIN INFUSED) |
| 0-2 | 38.5 | 0-6 | 18.8 | 0-6 | 36.7 |
| 2-16 | 23.0 | 6-18 | 18.3 | 6-18 | 20.3 |
| 16-40 | 0.0 | | | | |
| Total excreted | 61.5 | | 37.1 | | 57.0 |

Observations at autopsy revealed no gross pathology, except for scar tissue and thrombi in some instances at the sites of bleeding and gelatin infusion as noted previously.

Histologic examination of the heart, coronary vessels, aorta, carotid arteries, jugular veins, and renal arteries showed, in some instances, changes attributable only to prior disease. There was no vacuolization or subendothelial edema of the arteries and arterioles. Sections of the trachea, lungs, stomach, intestines, spleen, gall bladder, adrenals, striated muscle, brain, meninges, and gonads likewise showed no changes attributable to gelatin.

The parenchyma of the liver was normal. Many of the Kupffer cells, however, in all gelatin-infused dogs contained sudanophile droplets. Those in the areas of the acini around the periportal fields were most affected. The Kupffer cells in nine of twenty-four control sections of liver tissue showed moderate fat staining. The Kupffer cells of human liver, according to Levine,[28] normally contain neutral fat. The Kupffer cells of one of the dogs from which a portion of the liver was removed prior to gelatin infusions were fat-free. Immediately after completion of the series of infusions, the Kupffer cells were loaded with sudanophile droplets. At autopsy, seven weeks later, the same condition obtained. The nuclei of the Kupffer cells appeared normal in all cases.

Histologic examination of the kidneys of gelatin-infused dogs showed, in some instances, slight departures from the normal. The epithelial cells of the tubules of the proximal convolutions were sometimes swollen and occasionally vacuolated. Since gelatin is cleared through the kidneys, it is conceivable that in some animals it may have been responsible for the slight pathology observed.

## CONCLUSIONS

A highly purified and highly degraded 5 per cent gelatin solution prepared especially for intravenous therapy in human subjects was well tolerated by dogs when given repeatedly for several weeks in amounts commensurate with the fluid lost by hemorrhage. The only morphological pathology which might be attributable to gelatin in this series of dogs was the presence or increase of sudanophile droplets in the Kupffer cells. The significance of such a change is not known. It appears that the function of the Kupffer cells was not materially altered. None of our dogs developed infection of the respiratory tract or at the sites of bleeding and infusion.

The vascular pathology described by Hueper[6] was not observed in our series. Although Morehead and Little[21] found degenerative and reparative changes in the vascular system of their gelatin-infused dogs, they concluded that the lesions were not due to gelatin since similar changes were present in their control series.

Of the blood constants determined, only the sedimentation rate was altered by gelatin. This phenomenon is common to all macromolecular substances and occurs also when plasma protein concentrations are elevated. Gelatin did not interfere with the formation of hemoglobin or plasma protein, both of which were partially depleted at each hemorrhage, when infused weekly for periods as long as three months.

Gelatin appears not to be stored in the liver or kidneys. A major portion is excreted by the latter. The fate of the balance is unknown.

## SUMMARY

1. Gelatin solutions prepared for intravenous therapy proved innocuous to dogs when administered intravenously following repeated massive hemorrhages over a period of several weeks.

2. Vascular pathology was absent. The presence or increase of sudanophile droplets in the Kupffer cells was the only morphologic change attributable to gelatin.

3. Gelatin did not interfere with the formation of hemoglobin or plasma protein and appeared not to be stored in the liver or kidneys. A major portion was excreted by the kidneys.

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# ATTEMPTS AT THE EXPERIMENTAL PRODUCTION OF ARTHRITIS

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A RELATIONSHIP of the endocrine system to the phenomena of arthritis has long been suspected. Even before the development of modern endocrinology, it was recognized that the menopause seemed to have a significant influence upon the development of hypertrophic or osteoarthritis, and the term arthritis of the menopause appears in the literature as far back as 1855.

Recently an attempt has been made by one of us (R. P.), in conjunction with Scull, to integrate known and suspected factors within the neuroendocrine field in the pathogenesis of rheumatic disorders.[1]

Efforts have been made to simulate in experimental animals the phenomena of arthritis in some of its manifestations by means of injection of hormonal material, by the ablation of certain hormone-yielding organs, and by other steps of the kind. Among the most significant studies in this connection have been those of the Silberbergs[2] who have succeeded in producing some of the pathologic picture of hypertrophic or osteoarthritis in experimental animals by injection of extracts of the pituitary. Another attempt of the same general nature has been made by Selye,[3] who describes the induction of a number of phenomena characteristic of the arthritic state as the result of the injection of desoxycorticosterone acetate with the exhibition of certain other concomitant influences.

Selye[3] reports that the administration of desoxycorticosterone acetate in comparatively large doses causes the formation of "Aschoff bodies in the heart and the presence of periarteritis nodosa, a condition occasionally seen in man following an attack of rheumatic fever. Since in addition choreiform twitches and a few rare cases of arthritis were encountered in desoxycorticosterone acetate treated animals, it may be said that all the elements of the complex rheumatic syndrome were reproduced in experimental animals."

He further states, "The arthritic lesions, which are rarely seen in the intact desoxycorticosterone acetate treated rat, develop with great frequency in similarly treated thyroidectomized or adrenalectomized animals, especially if these are kept in cold surroundings."

He also says, "Previous experiments revealed that unilateral nephrectomy and sodium chloride treatment increase the sensitivity of rats to many overdosage effects of desoxycorticosterone acetate." In his experiments "all animals were sensitized by ablation of the left kidney 5 days before injections were started and given 1% NaCl to drink beginning on the day of operation."

It is obvious that importance attaches to conclusions of this nature and it was therefore decided to repeat, so far as possible, Selye's experiments.

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*Experiment 1 (Divided Into Three Groups).—*

*Group A:* Eight female albino rats weighing 20 grams each were put on Fox chow plus 1 per cent NaCl to drink, and six of them were given injections of 3 mg. desoxycorticosterone acetate* twice daily for forty-two days. To serve as controls, two received no injections. The temperature of the animal room varied from 55 to 80° F. Three of the six injected rats showed some redness and swelling of the hind feet and knee joints eleven to eighteen days after injection. This subsided and reoccurred from time to time, disappearing before autopsy. The two controls showed no gross lesions. At autopsy six of the eight rats, including both controls, were found to have parasitic infection of the livers, the parasite being a strobilocercus of the *Taenia Taeniaformis*, a common

Fig. 1.—One articular surface of experimentally induced arthritis shows focal destruction of cartilage and underlying bone. The normal tissues are replaced by seminecrotic material, proliferating cartilage, and fibroblasts.

tapeworm of the domestic cat, the intermediate host being rats and mice. Sections were made of the hind legs and feet. The three affected rats showing gross swelling revealed microscopic joint changes. These were slight proliferation of the capsule with the lining of the cells piled up over a small ulcer in some places; loss of cartilage; a proliferation of fibroblasts replacing the ulcerated cartilage; marked focal destruction of articular cartilage on both articular surfaces; and adhesions extending from one ulcerated area to the other with loose connective tissue replacement (Fig. 1).

*The desoxycorticosterone acetate used in these experiments was made available through the courtesy of Dr. Edward Henderson, Director, Division of Clinical Research, Schering Corporation, Bloomfield, N. J.

Of the two control rats receiving no injections, one leg joint of one rat showed one-third of the joint surface of the cartilage to be replaced by irregular masses of proliferating connective tissue.

*Group B:* A similar group of eight rats was given Fox chow and plain water to drink and was injected identically with Group A, in the same room, at the same time, for the same length of time, to determine whether the absence of 1 per cent salt made the animals more or less susceptible to arthritis. There was never any gross redness or swelling. One injected rat died. One control was not sacrificed. At autopsy, of the five injected rats and one control, three, including the control, were found to be infected with parasites. Sections of the hind legs were made and only one of the injected rats showed any joint change. In this animal one joint surface showed a necrotic area in the cartilage.

Within the joint cavity of another joint, seminecrotic tissue was observed to be attached to the synovia. The control rat was negative.

*Group C:* At the same time eight rats varying in weight from 25 to 86 grams were put on Fox chow plus 1 per cent NaCl, and four were given injections. The remainder served as controls. All were placed in a room without heat where the temperature was 40 to 55° C. The four injected rats died in a few days and four more, weighing 116 to 122 grams, were substituted. The controls which did not succumb weighed 82 to 86 grams. The experiment was discontinued after thirty-five days, since there was no gross redness or swelling. At autopsy no liver parasites were found. One control rat and the four injected rats were sectioned. One test rat showed proliferation of connective tissue from the synovia into one joint cavity.

It may be concluded from this experiment that:

1. Young rats, overdosed with desoxycorticosterone acetate and given 1 per cent NaCl to drink, showed joint changes in 50 per cent of the experimental animals, somewhat resembling certain phases of hypertrophic or osteoarthritis found in man. Some joint change of similar nature was seen in one joint of one control.

2. The subjection of experimental rats to environmental cold, as described, does not substantiate Selye's findings, since no evidence of gross swelling or crippling resulted.

3. The rats not ingesting salt showed no marked incidence of gross or microscopic changes in the joints.

*Experiment 2.*—This experiment was carried out on mononephrectomized and bilateral adrenalectomized rats. The following steps were employed in each operative procedure:

1. The rat was anesthetized with ether.

2. The rat under anesthesia was secured to an operative board of soft wood by means of rubber bands looped about the feet and over hooks which were attached to the board.

3. The operative area was clipped with scissors and then shaved.

4. The skin of the operative site was painted with tincture of metaphen.

5. Sterile draping of the operative site was performed.

6. The surgeon used sterile gloves after thoroughly scrubbing his hands with soap and water.

7. Sterile instruments were used throughout.

8. After the operation had been completed, the rat was kept in a warm cage until out of anesthesia.

The surgical technique employed in each experiment is now given in detail.

*Technique of Unilateral Nephrectomy and Bilateral Adrenalectomy:* Ether anesthesia was used and the rats were placed in a prone position. A dorsal midline incision was made through the skin at the level of the kidneys. The subcutaneous fascia was incised on the right, exposing the muscle. A longitudinal incision was then made along the lateral border of the right dorsal muscle mass. The muscle was retracted medially and the kidney and adrenal exposed. The right kidney and adrenal were delivered. The kidney pedicle was ligated with No. 00 plain catgut and the kidney and adrenal excised. Similar exposure was then made on the left side. The kidney was delivered and the adrenal was displaced upward, detached from the upper pole of the kidney, and removed. The kidney was returned to its anatomic position. The skin was then closed with interrupted fine silk sutures.

Thirteen female albino rats weighing from 40 to 50 grams were mononephrectomized, and seven of these were bilaterally adrenalectomized. They were given 1 per cent NaCl to drink and Fox chow. Five days later, weighing 65 to 90 grams, they were given injections of 3 mg. of desoxycorticosterone acetate twice daily for twenty-eight days. This was given to the seven adrenalectomized, mononephrectomized rats and to four of the six mononephrectomized rats. The mononephrectomized and one intact rat were kept as controls, receiving no injections. They were all kept in a warm room.

No redness or gross swelling was observed. At autopsy none of the rats showed parasitic infestation. The hind legs were examined microscopically with the following results:

1. Two of the seven adrenalectomized, mononephrectomized rats showed some joint changes.

2. One of the four mononephrectomized rats developed a joint showing practically complete obliteration of the joint cavity due to adhesions of adult connective tissue between both articular surfaces. The same joint showed destruction, and connective tissue replacement, of articular cartilage.

3. One control rat, nephrectomized, showed slight absorption of cartilage and slight proliferation of endothelial cells at the attachment of the synovia.

It is evident from these experiments that the operations discussed did not contribute further to the development of arthritis. Normal rats overdosed with desoxycorticosterone acetate showed more evidence of joint changes than did those operated upon.

*Experiment 3.*—Eight rats were mononephrectomized and thryoidectomized as follows:

The previously described technique for unilateral nephrectomy was observed. The animal was then placed in the dorsal position for *thyroidectomy.* The head of the animal was kept in extension by means of a rubber band looped over the upper incisors. A ventral midline incision was made in the neck. The salivary glands were freed at the midline and retracted laterally. The sternohyoid muscle was divided in the midline. The thyroid was exposed by careful, blunt dissection with a small curved hemostat. The gland was grasped with

tooth forceps, and while retraction was exerted upward and laterally the gland was dissected free from its attachments by means of a needle. Bleeding was negligible when the gland was removed by this technique. The skin was then closed with interrupted fine silk.

Five days after operation, the eight rats were injected with 3 mg. of desoxycorticosterone acetate twice daily for a total of ninety injections.

None of these rats showed any gross redness or swelling. One died at five weeks. Since these rats showed no gross evidence of arthritis, it was decided to add one more stress in the form of an injection of bacterin or bacterial toxin. This, when given to human beings, often causes a systemic reaction such as chills and fever. With this thought in mind, it was decided to subject these operated rats, overdosed with desoxycorticosterone acetate, to increasing doses of whole typhoid bacterin and serobacterin.

The remaining seven rats were divided into three groups:

Two rats were given .3 ml. of typhoid whole bacterin.

Three rats were given .3 ml. of serobacterin typhoid.

Two rats were not given any bacterin, being left as controls.

Two normal rats were used as controls on the bacterin alone, one receiving the serobacterin, the other, the whole bacterin. The doses of bacterin were given at five-day intervals and increased to 1 ml., the schedule of dosage being .3, .6, .8, 1 ml., and 1 ml. Since these injections did not seem to have any gross effect on the health of the rats, all were sacrificed and autopsied.

The two control rats, receiving typhoid bacterin *only*, showed no joint changes.

Of the three remaining rats, only one of which had received typhoid bacterin, all showed extensive ulcerations and connective tissue replacement of articular cartilage by fairly adult connective tissue.

The two control rats, receiving typhoid bacterin *only*, showed no joint changes.

This experiment with mononephrectomized, thyroidectomized rats subjected to overdosage of desoxycorticosterone acetate and the addition of typhoid bacterin did not increase the extent or incidence of joint changes.

*Experiment 4.*—Another group of fifteen rats weighing 30 grams was treated as follows:

Seven were mononephrectomized as previously described, and ovariectomized.

Four were ovariectomized. In one the testicles were removed.

*Technique of Bilateral Oophorectomy:* Ether anesthesia was used and the rats were placed in a prone position. The right ovary was exposed through the thin muscle wall just below the dorsal muscle mass. Hemostasis was then insured by ligation of the upper horn of the uterus on the right side with No. 00 plain catgut. The right ovary and the oviduct were then removed. A similar technique was used to remove the left ovary and oviduct. The skin incision was closed with interrupted fine silk sutures.

Three of the fifteen rats were left intact as controls. All were put on Fox chow and 1 per cent NaCl. Five days after operation all were given 3 mg. of desoxycorticosterone acetate twice daily for thirty days, a total of sixty injections.

The rats gained weight and showed no gross evidence of arthritis. All were sacrificed and autopsied. There was no parasitic infection of the livers.

One rat which had a stitch abscess for twenty days before the infected stitch was removed and the lesion healed was watched especially to see if it would show any joint involvement due to the added stress of infection; it did not, however, and microscopic findings of its joints were negative.

The results of this experiment were:

Three of the seven mononephrectomized, gonectomized rats had some minor changes of the cartilage and underlying bone of the leg joints.

Two of the five ovariectomized rats had a few small foci of erosion of articular cartilage extending down to the bone.

Two of the three control rats, having no operations, showed focal ulcerations or erosion of cartilage in some places extending down to the bone.

This experiment reveals that the operative procedures of mononephrectomy and ovariectomy, or ovariectomy alone, do not lead to joint changes resembling "rheumatic and rheumatoid conditions in man" with a frequency greater than in normal rats which are unoperated and overdosed with desoxycorticosterone acetate.

Since the controls in these experiments showed some joint change, a food experiment was conducted.

*Experiment 5.*—Twenty male and twenty female rats, most of them weighing 30 to 50 grams, a few weighing up to 100 grams, were divided into four groups, five of each sex of varying weight in each group. Both sexes were included to ascertain if one sex were more prone to arthritis than the other.

Group 1 was given Fox chow plus 1 per cent of NaCl.

Group 2 was given Fox chow plus 2 per cent of NaCl.

Group 3 was given Fox chow plus plain water.

Group 4 was given no Fox chow, but bread, greens such as lettuce or celery, and water.

All groups were kept on schedule during the summer months, from June 15 to September 26, when they were sacrificed with the following results:

1. None of the rats at any time showed any gross evidence of arthritis.

2. All rats of the group on 2 per cent NaCl were stunted in growth, scaly, dirty, and emaciated. They gained weight, but slowly.

3. Twenty-three of the thirty rats eating Fox chow showed liver parasites.

4. None of the rats, male or female, of Group 1 or 2 showed any microscopic joint changes.

5. Only three of Group 3 showed any microscopic joint change, this being found in female rats only. This joint change is described as fairly extensive destruction of articular cartilage with adhesions between articular surfaces.

6. Group 4, however (not on Fox chow), showed no liver parasites, was grossly healthy, and gained weight at a normal rate, but microscopically six of seven presented joint changes.

These joint changes constituted the most marked microscopic findings of the entire group of experiments undertaken.

The changes consisted of a fairly extensive destruction of articular cartilage, ulceration, and adhesions connecting the ulcerating surfaces (Fig. 2).

Finally, reviewing all the variations carried out in these five experiments and the results obtained, the work was concluded by repeating the first experiment.

Fig. 2.—One articular cartilage, in experimentally induced arthritis, shows a superficial ulcer in which the cartilage cells have been replaced by seminecrotic material.

*Experiment 6.*—Rats of our own raising, weighing 30 grams, were used. Several were sacrificed before beginning the experiment to see if they were free of liver parasites. The Fox chow was sterilized, as were all the cages, to eliminate, if possible, parasitic infestation.

It was decided to use two preparations of desoxycorticosterone acetate; a 3 mg. emulsion of crystalline desoxycorticosterone, and a 5 mg. in 1 ml. of peanut oil.

Sixteen rats weighing 30 grams were used. Eight were given 3 mg. of the emulsion twice daily. Two were kept as controls with no injections. Six were given .5 ml. intramuscularly twice daily of the oil preparation one dose in the muscle of each hind leg. All were given sterilized Fox chow and 1 per cent NaCl for drinking water. The eight rats received injections for a period of thirty-nine days, at which time injections were discontinued.

There was no gross evidence of arthritis in either test or control rats, and all were sacrificed twenty-seven days later. The six rats on oil preparation showed much edema of the hind legs and feet which was believed to be unabsorbed oil.

The rats received a total of 18 ml. per rat over a period of twenty-four days. The day after the last injection one of these rats was sacrificed and

about 7 ml. of oil were aspirated from the tissue of the hind legs and the peritoneal cavity. The remaining animals were kept on their food, plus 1 per cent salt solution for thirty-three days longer to allow for further absorption of oil and subsidence of edema.

At autopsy no liver parasites were found. Eight of the sixteen rats, one of which was a control, or 50 per cent, showed focal destruction of cartilage and ulceration of cartilage with loose connective tissue stretching across the joint cavity from ulcer to ulcer. Some of the underlying bone was seminecrotic in the oil-treated rats (Fig. 3). These joint changes were most marked in the oil-treated animals.

Fig. 3.—Focal destruction or ulceration of cartilage and underlying bone on opposing articular surfaces in experimentally induced arthritis. The ulcerated areas are joined by a band or adhesion of young connective tissue which stretches across the joint cavity.

## COMMENT

It is accepted that so-called spontaneous arthritis occurs sometimes in experimental animals for reasons that are not clear. In an attempt to meet this situation efforts were made to eliminate the influence of parasitic infection which was encountered in Experiments 1 and 2. By the same token, dietetic experiments were conducted to show that experimental rats on a balanced diet do not show the arthritic changes encountered in the rats fed an unbalanced diet. Evidence has been adduced that there may be an apparently spontaneous occurrence of arthritis in rats due to a "pleuro-pneumonia-like" organism.[4] Normal animals, however, may also harbor this organism. The animals, in the series here reported, that showed arthritis gave no clinical evidence of systemic illness and autopsy revealed no pneumonitis or other evidence of pulmonary disease.

It is further to be observed that as far as so-called spontaneous arthritis is concerned, a chief conclusion of this work has to do with failure to reproduce arthritis as the result of certain operative procedures followed by administration of desoxycorticosterone acetate.

## SUMMARY

Arthritic-like lesions were encountered in groups of rats maintained on standard laboratory rations and on grossly unbalanced diets and in animals receiving injections of massive doses of desoxycorticosterone acetate. In one group receiving massive doses of desoxycorticosterone acetate, together with a one per cent NaCl solution for drinking water, joint changes were formed in 50 per cent of young female white rats. These results are at variance with the findings of certain other workers that arthritic lesions are rarely seen in intact desoxycorticosterone-treated rats. Attempts to increase the incidence and severity of these arthritic-like lesions by exposure to cold, by thyroidectomy, adrenalectomy and gonadectomy were unsuccessful. The changes observed in the joints are those of focal areas of degeneration and ulceration in the articular cartilages.

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# STUDIES ON THE NEPHROTOXIC ACTION OF *dl*-SERINE IN THE RAT

## III. The Influence of Experimental Hydronephrosis

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EXTENSIVE renal necrosis in the kidneys of young male rats has been shown to be caused by *dl*-serine ($\alpha$-amino-$\beta$-hydroxy propionic acid).[1-3] The protective action of various amino acids and related compounds against the damaging action of *dl*-serine[4, 5] was reported in a previous communication. *dl*-methionine and glutathione exert a considerable protective influence. This is probably not due to SH-groups since cysteine and thioglycolic acid have only small beneficial effect and 2.3-dithiopropanol (BAL) has none. *dl*-alanine, glycine, *dl*-threonine, glycolic acid, butyric acid, and pyruvic acid have a marked protective influence. *l* (+) histidine monohydrochloride and lactic acid afford appreciable protection, and *l* (+) arginine monohydrochloride and *dl*-valine, moderate protection. Some influence was seen from *l* (+) glutamic acid, while glucose, sodium acetate, and sodium chloride were without effect. It is postulated that the protective action of these substances is due to their competitive suppression of tubular reabsorption of the injurious *dl*-serine. The present report deals with the nephrotoxic effect of *dl*-serine in the presence of hydronephrosis of one kidney.

### METHOD

Sixteen adult male albino rats from the Wister strain were used. Their weight varied between 195 and 360 grams. Adult male animals were previously found to be as susceptible as young rats to the nephrotoxic action of *dl*-serine.[6] The left ureter was ligated under sodium amytal anesthesia. *dl*-serine was injected 1, 3, 4, 6, 7, 8, 9, 12, 24, 40, 64, 88, 112, and 136 hours after ligation of the ureter. One hundred milligrams of *dl*-serine dissolved in 3 ml. of distilled water were given for each 100 grams of weight by intraperitoneal injection. The animals were sacrificed twenty-four hours later. Tissues were fixed in formalin and stained with hematoxylin-eosin. Alkaline phosphatase activity was demonstrated with Gomori's method[7] as modified by Kabat and Furth[8] in tissues fixed in cold acetone. The incubation time in the substrate mixture was two hours. No counterstain was used.

### RESULTS

In all animals the kidneys with uninterrupted urinary flow showed extensive necrosis in the region of the cortico-medullary junction. On gross examination a small, irregularly shaped strip of grayish discoloration at the innermost portion of the cortex indicated the necrotic area (Fig. 1). The epithelium of many tubules in this area was almost completely destroyed (Fig. 2). With

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the help of vital staining with lithium carmine and by the maceration and dissection technique of Oliver,[9] the kidney damage was localized to the terminal segments of the proximal convoluted tubules.[6] There was no significant difference in the extent of renal necrosis in the obstructed as compared with the unobstructed kidney when *dl*-serine was given within three hours after the ligation of the ureter. In the kidneys that had been obstructed from four to seven hours, the extent of the necrosis was considerably decreased. In one of the experiments, only few necrotic tubules were seen in the hydronephrotic kidney of a rat injected with *dl-serine* six hours following the ligation of the ureter (Fig. 3). In all animals in which *dl*-serine was given eight or more hours

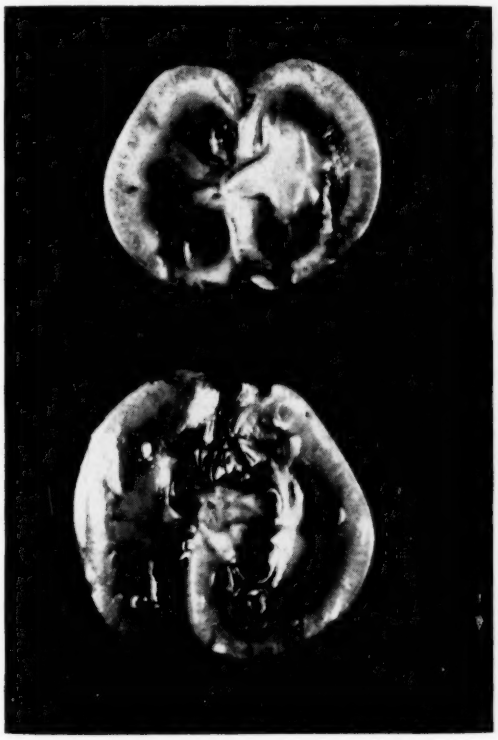

Fig. 1.—The kidneys of a male rat weighing 210 grams which was given 210 mg. *dl*-serine by intraperitoneal injection six hours after ligation of the left ureter. The animal was sacrificed twenty-four hours later. The unobstructed right kidney (top) shows an irregularly shaped strip of discoloration at the cortico-medullary junction, indicating the necrotic area. The left kidney (bottom) shows hydronephrotic dilatation without the necrotic area seen in the other kidney. Approximate magnification, ×2.

following the obstruction of the ureter, a similar degree of almost complete protection in the obstructed kidney took place. Only very occasional tubules were necrotic. The kidneys in which the ureter had been ligated showed the typical gross and microscopic changes of experimental hydronephrosis increasing in intensity with the duration of the experiment.[10]

Phosphatase activity was normal in animals which were killed within thirty-six hours after the ureter had been ligated. Considerable decrease in phosphatase occurred in the remaining animals in which the obstruction had

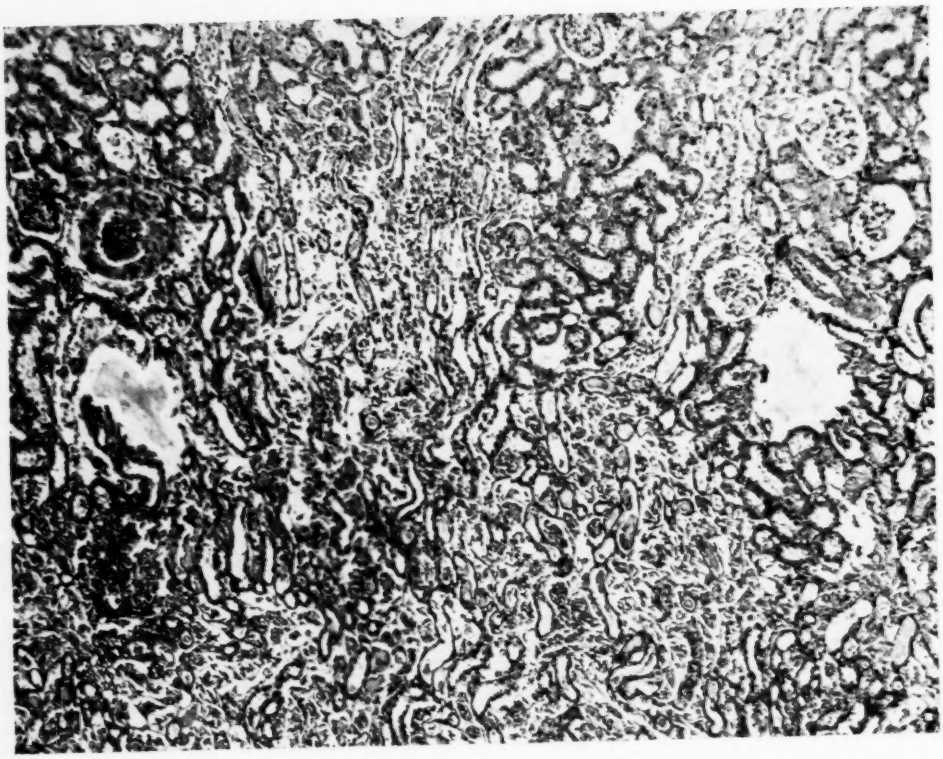

Fig. 2.—Microscopic section of the right unobstructed kidney pictured in Fig. 1. Extensive necrosis involving the distal portion of the proximal convoluted tubules is seen. Hematoxylin-eosin ×150.

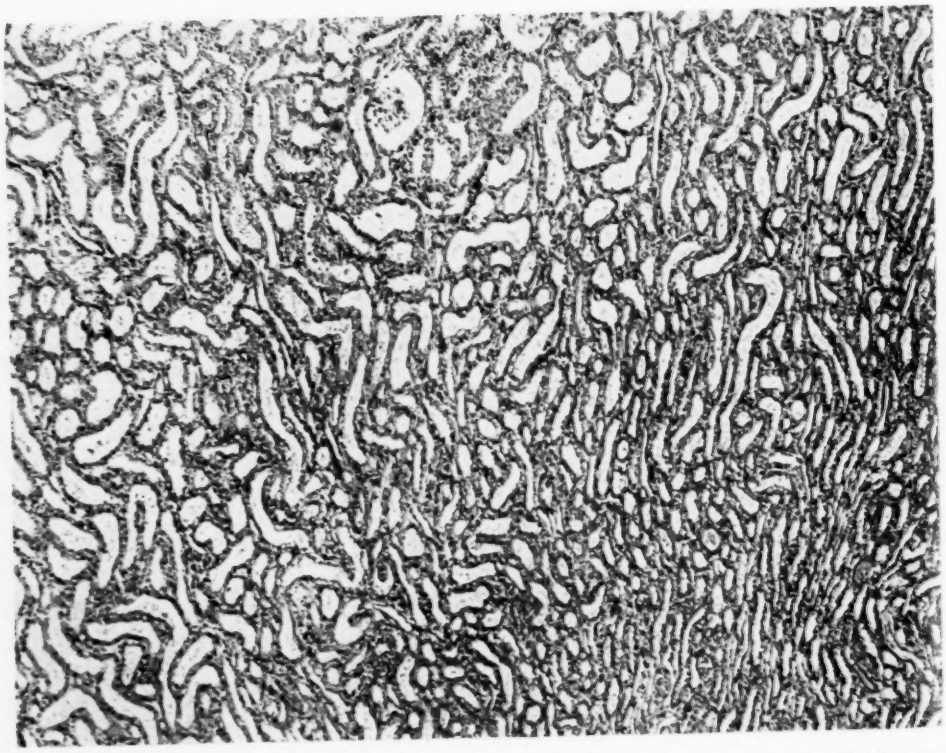

Fig. 3.—Microscopic section of the left obstructed kidney pictured in Fig. 1. Fairly generalized dilatation of the cortical tubules is seen. Renal necrosis is almost completely absent. Hematoxylin ×150.

lasted for a longer time. The necrotic cells in the damaged proximal convoluted tubules of the unobstructed kidney revealed phosphatase activity as previously described.[6]

## COMMENT

Depression of the nephrotoxic action of mercury bichloride as early as fourteen to twenty-four hours following ligation of the ureter was described by Elbe[11] in 1905. Elbe's findings were later confirmed by Kosugi.[12] Frola found a similar protective influence of hydronephrosis on the damaging effect of uranium nitrate.[13] These observations were recently extended by Willmer[14] who confirmed the protective effect of experimental hydronephrosis upon the nephrotoxic action of uranium nitrate and mercury bichloride. Sucrose,[15] however, as well as racemic tartaric acid and diethylene glycol produced the same amount of hydropic degeneration in the cortical tubules of the hydronephrotic and the normal kidney. In rabbits in which glomerulonephritis had been induced with duck-antirabbit serum, after the ureter in one kidney had been ligated, Reubi[16] observed nearly complete protection in the obstructed kidney. In this connection the observation of Fahr[17] is of interest. He found at autopsy on a 37-year-old woman severe subacute glomerulonephritic changes in one kidney, while the other kidney with hydronephrotic atrophy showed none.

As early as three hours following obstruction of one ureter there occurred appreciable diminution in toxicity, and six to seven hours following obstruction almost complete elimination of the injurious effect of *dl*-serine on the hydronephrotic kidney. The short time of obstruction necessary to modify the nephrotoxic action of *dl*-serine is more significant for this substance may cause microscopically demonstrable kidney changes within thirty minutes. These changes become extensive within the next few hours.[6]

No significant decrease in phosphatase activity is seen in the very early stage of hydronephrosis. Decrease, however, is noticeable in the hydronephrotic kidneys in the next few days and becomes progressively more pronounced. This is in full agreement with Willmer's findings.[19] Lipase activity in the proximal convoluted tubules of the hydronephrotic kidney shows a similar behavior.[20] While, therefore, the decrease in these enzymatic activities is apparently a comparatively late sign of disturbed cellular function, there are other evidences of very early changes.

Slight general dilatation of the tubules takes place within three to six hours following ligation of the ureter.[21, 22] Not only dilatation extending into the proximal convoluted tubules, but also flattening of epithelial cells and considerable parenchymatous and vacuolar degeneration in the cytoplasm occur within short time after ligation of the ureter.[10, 12] In acute experiments in which the ureter of one kidney is obstructed and the urine of both kidneys is simultaneously analyzed, a decreased concentration of nitrogenous constituents and electrolytes is found.[23, 24] The excretion of injected indigo carmine is considerably reduced as early as two to four hours following ligation of the ureter.[23, 25]

Amino acids pass the glomerular filter and are reabsorbed in the convoluted tubules.[26] In view of the fact the glomerular filtration in experimental hydro-

nephrosis[27] is probably undisturbed for some time, an impaired passage of amino acids through the glomeruli is not likely, although not impossible.[28] It is reasonable to assume that early functional disturbances in the convoluted tubules are responsible for the slowing up of the reabsorption of the toxic *dl*-serine. Apparently the *dl*-serine is not concentrated in the distal portion of the proximal convoluted tubules to such an extent as to permit it to exert its destructive action.

Convoluted tubules occasionally show typical necrosis even after several days of experimental hydronephrosis. A similar phenomenon was noticed previously with mercury bichloride and uranium nitrate.[12, 14] That even after several weeks of ligation of the ureter the convoluted tubules have not completely lost the ability to concentrate injected lithium carmine has been shown by Suzuki.[29]

## SUMMARY

In adult male rats the ureter of one kidney was ligated, and in intervals of 1 to 136 hours following the obstruction of the ureter *dl*-serine was injected. The necrotizing nephrosis induced by *dl*-serine is appreciably reduced in kidneys in which the ureter has been ligated for three to seven hours. It is almost entirely suppressed if the injurious amino acid is given eight or more hours after the ligation of the ureter. It is assumed that early disturbance of tubular reabsorption does not permit the concentration of *dl-serine* in the sensitive cells of the proximal convoluted tubules to become sufficiently great to exert its destructive action.

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# LABORATORY METHODS

## A METHOD FOR SHOWING ANTIBACTERIAL ACTIVITY OF MINUTE QUANTITIES OF GRAMICIDIN OR PENICILLIN

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THE test described is based on the method of Jones and Simms[1] who showed that bacteriostasis could be measured as a function of inhibition of colony size and hemolytic zones produced by Group A streptococci on blood agar plates.

Spingarn and King[2] used a similar procedure in studing the comparative susceptibility of Group A streptococci to penicillin.

By the use of this method we have been able to compare the activity of gramicidin and penicillin on a weight basis, and to determine the gramicidin content in tyrothricin samples. The following examples give an indication of the results obtained.

### METHODS

An eighteen-hour blood broth culture of C203 (Group A) streptococcus is diluted to $10^{-5}$ in plain broth.

Dilutions of the drug to be tested are made in a suitable diluent, that is, penicillin in water, or tyrothricin or gramicidin in 50 per cent propylene glycol-water.

Sterile defibrinated rabbit blood (taken from rabbits which had been starved for twenty-four hours prior to heart puncture) was used. We have routinely used blood which has been at least twenty-four hours old and not more than five days old.

The tests are set up as follows: To Petri dishes (100 by 15 mm.) add:

0.5 ml. defibrinated rabbit blood
0.1 ml. $10^{-5}$ culture dilution
0.1 ml. drug dilution (do not mix with blood or culture until agar is added)
11.8 ml. proteose-tryptone agar melted and cooled to 45°C.

The contents of the plates must be thoroughly mixed to insure an even distribution of all components.

Various amounts of a drug in blood agar are made to give an adequate range of concentrations. This range should cover from 80 to 10 per cent inhibition of growth. Usually five plates are prepared to study one sample. Four of these contain different concentrations of the drug in blood agar, and one

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(containing no drug) serves as a control. This last one gives maximum hemolytic zones around each colony.

After twenty-four hours' incubation at 37°C., the diameters of the hemolytic zones are measured with an ocular micrometer and a mechanical stage micrometer. The longest diameters of the hemolytic zones around five representative colonies are measured and averaged.

Streptococcus C203, under the conditions employed, usually gives a hemolytic zone 2.0 mm. in diameter. Smaller colonies and hemolytic zones are indications of the activity of the drug under test. To give a figure which increases with the activity, the "inhibitory activity" is expressed as

$$100-100\frac{(\text{mm. of inhibitory zone with drug})}{(\text{mm. of inhibitory zone with no drug})}.$$

Table I gives ranges of concentrations of gramicidin and crystalline penicillin per milliliter of blood agar which show differences that may be measured and plotted. The results shown are from duplicate assays on two samples of gramicidin and two batches of crystalline penicillin.

TABLE I. INHIBITION OF HEMOLYTIC ZONES OF STREPTOCOCCUS C203 IN BLOOD AGAR BY GRAMICIDIN AND PENICILLIN

| DRUG | INHIBITORY ACTIVITY* CONCENTRATION μG DRUG PER ML. BLOOD AGAR | | | | |
|---|---|---|---|---|---|
| | 0.006 | 0.004 | 0.002 | 0.001 | 0.0008 |
| Gramicidin | | | | | |
| 15975 | | 50* | 25 | | 5 |
| 15975 | 80 | 50 | 25 | 11.5 | |
| 23-19-B | | 50 | 25 | 13 | 8 |
| 23-19-B | 70 | 40 | 20 | 10 | 7.5 |
| Crystalline penicillin CSC | | | | | |
| (1) | | 60 | 40 | 22 | 18 |
| (2) | | 55 | 35 | 18 | |

*Inhibitory activity defined as $100-100\frac{(\text{mm. inhibitory zone with drug})}{(\text{mm. inhibitory zone with no drug})}$.

It is seen in Table I that detectable inhibition can be shown by gramicidin and penicillin when present in amounts as small as 0.8 millimicrogram per milliliter of blood agar. The results appear to be reproducible as shown by the duplicate figures. On a weight basis, penicillin appears to be more active than either of the gramicidin samples.

Tyrocidine fails to inhibit the growth of streptococcus C203 on blood agar plates when present in a concentration of 1 microgram of blood agar. Thus the activity shown by tyrothricin samples may be referred to as being a function of the gramicidin present.

By comparing samples of tyrothricin with a standard of known gramicidin content, it is possible to judge the gramicidin content of the unknown as greater than or less than the known standard. Table II shows such comparisons.

Sample 189 (figures show triplicate measurements) was a standard containing 20 per cent gramicidin, 80 per cent tyrocidine HCl by weight.

Sample 85-15 contained 15 per cent gramicidin and 85 per cent tyrocidine HCl by weight.

The other nine samples were from lots of tyrothricin which had been shown by the Dubos[3] albumin broth test (streptococcus H69D) to contain more than 20 per cent gramicidin when compared with a known 20 per cent gramicidin control.

Table II shows reasonable reproducibility of the 20 per cent gramicidin control sample (189) in triplicate tests, and, as expected, considerably lower values are shown by the 15 per cent gramicidin sample (85-15).

TABLE II. INHIBITION OF HEMOLYTIC ZONES OF STREPTOCOCCUS C203 IN BLOOD AGAR BY TYROTHRICIN

| TYROTHRICIN SAMPLE | GRAMICIDIN CONTENT (%) | INHIBITORY ACTIVITY* CONCENTRATION μG DRUG PER ML. BLOOD AGAR | | |
|---|---|---|---|---|
| | | 0.04 μg | 0.02 μg | 0.008 μg |
| | 20 | 80* | 50 | 21.5 |
| 189 | 20 | 80 | 46 | 22 |
| | 20 | 80 | 47 | 20 |
| 76 | ? | | 55 | 25 |
| 58 | ? | | 56 | 30 |
| 56 | ? | | 55 | 28 |
| 70 | ? | | 80 | 60 |
| PD | ? | | 65 | 45 |
| 69 | ? | | 60 | 40 |
| 75 | ? | | 55 | 30 |
| 73 | ? | | 58 | 28 |
| NP | ? | 70 | 45 | 21 |
| 85-15 | 15 | 60 | 37 | 5 |

*Inhibitory activity defined as $100-100\frac{\text{(mm. inhibitory zone with drug)}}{\text{(mm. inhibitory zone with no drug)}}$.

All of the other samples show a greater inhibitory value than the 20 per cent gramicidin control samples. Samples 70, PD, and 69 give particularly high values showing 60, 45, and 40 per cent inhibitory activity at the level of 0.008 μg per milliliter of blood-agar.

It is of interest to note that an inhibitory value of approximately 50 per cent is given by 0.02 microgram per milliliter of the standard (20 per cent gramicidin, 80 per cent tyrocidine) sample (Table II) as compared with a 50 per cent inhibitory activity by 0.004 microgram per milliliter by crystalline gramicidin (Samples 15975 and 23-19-B, Table I).

## SUMMARY

A method is described by which the inhibitory effects of millimicrogram quantities of penicillin or gramicidin can be measured.

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# A STREAK PLATE METHOD FOR DETERMINING GROWTH CURVES

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WITH THE TECHNICAL ASSISTANCE OF ANNE GUNTHER

WHILE investigating the action of certain sulfur suspensions on the growth of *Staphylococcus aureus*,[1] a technique was developed which was far less laborious than the pipette dilution, pour plate method for demonstrating growth curves. By adherence to certain details of technique, remarkably reproducible graphs were obtained for the first eight hours of growth.

## METHOD

*Test Culture.*—The first essential is to keep the inoculum within the range of the method. A three and one-half hour culture of *Staph. aureus* (Oxford strain) in bacto nutrient broth was prepared one day before each test. Two loopfuls of a 1:20,000 dilution were spread separately on two halves of an agar plate to estimate the population. The culture was then left overnight in the icebox.

*Agar.*—Twelve to fifteen cubic centimeters of bacto nutrient agar (1.5 per cent agar) were poured into Petri plates which were stored three to four days at 37° C. to dry. This was necessary to avoid condensation on the surface of the agar which interferes with accurate bacterial counts.

*Loop.*—A standard loop of nichrome wire, which when filled held approximately 0.002 c.c. of broth, was employed. In making subcultures the tube containing the test material was held in a slightly slanted position and only the loop immersed, taking care to pick up the maximum amount of fluid. Recently Calkins[2] has shown that under specified conditions the quantity of fluid taken up by a loop is remarkably constant.

*Spreading a Loopful of Fluid.*—The dry agar plates were ruled horizontally with a glass pencil, the size of the area between the lines being proportional to the expected bacterial count. (When large counts are expected, a whole plate is used for one preparation.) Then one loopful from each test preparation was spread evenly over the entire surface of the agar marked out for it, care being taken to avoid cutting into the agar with the loop. It is, of course, important not to leave even tiny droplets of test fluid unspread.

*The Bacterial Count.*—The colonies were counted after eighteen to twenty-four hours' growth at 37° C. The figures obtained were multiplied by a factor of 500 to convert them to bacteria per cubic centimeter, since one loopful held 0.002 c.c. of culture. It is obvious that negative cultures might be obtained from cultures that actually contain a few organisms per cubic centimeter.

When an entire plate was used, at least 200 individual colonies (representing 100,000 organisms per cubic centimeter) were counted. When properly inoculated, none of the colonies run into one another (see Plate I).

In the first eight hours of growth, the period of interest in our present study, the number of organisms in one loop was rarely more than 200. Therefore, the graphs represent the actual number of organisms in undiluted cultures, calculated to 1 c.c. volume. When the counts were higher than 200 colonies per plate, rough estimates were made and recorded, such as 1 plus for 200 to 400 colonies; 2 plus for 400 to 600 colonies; 3 plus for 600 to 800 colonies; and 4 plus for over 800 colonies.

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The control tube was usually slightly cloudy after four hours' growth, and always definitely cloudy after six hours. In such preparations the count was always in the millions and, therefore, out of the range of this technique.

One possible source of error was that a loopful of test fluid, especially from the higher concentrations of an antibacterial agent, might have carried over sufficient material to inhibit growth. To explore this possibility, in several of our experiments one loop was spread over agar as usual, and a second loop from the same tube was mixed with melted and cooled agar for pour plates. We found that the two sets of counts were essentially alike and therefore concluded that the possibility of this error in our bacterial counts was no greater than the possibility of error with the pour plate method.

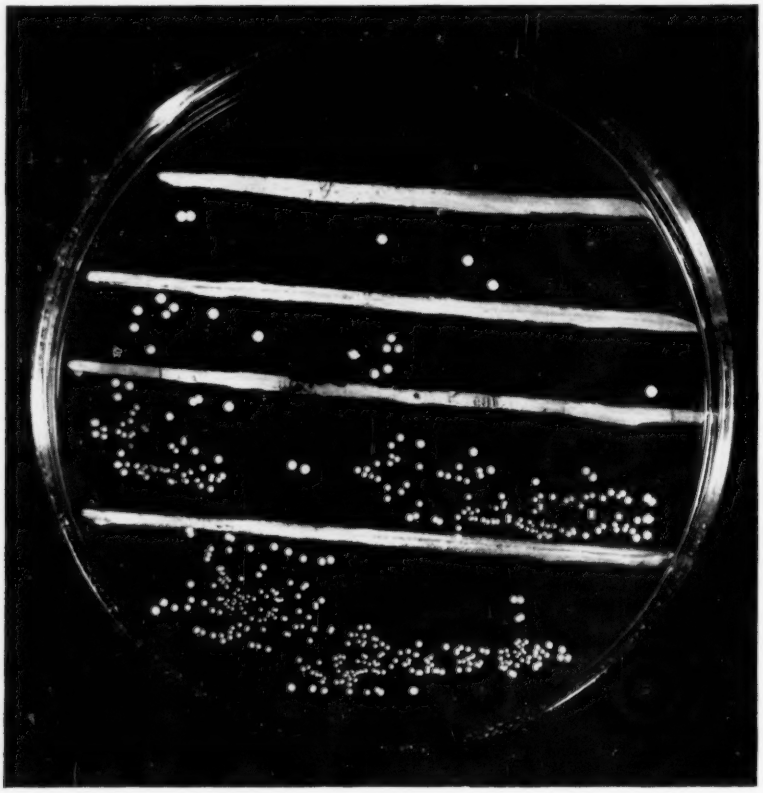

Plate I.*—Antibacterial effect of penicillin upon the growth of *Staph. aureus*. Streak cultures of test mixture dilutions made on agar after two hours' incubation at 37° C.

| READING DOWNWARD | COLONY COUNT | CALCULATED NUMBER OF ORGANISMS/C.C. |
|---|---|---|
| 1. Penicillin content 0.1 u./c.c. | 0 | 0 |
| 2. Penicillin content 0.075 u./c.c. | 5 | 2,500 |
| 3. Penicillin content 0.05 u./c.c. | 14 | 7,000 |
| 4. Penicillin content 0.025 u./c.c. | 130 | 65,000 |
| 5. Penicillin content 0.01 u./c.c. | 190 | 95,000 |

The initial number of organisms per c.c. was 27,500.

The control test after two hours' incubation at 37° C. contained over 100,000 organisms per cubic centimeter.

## TEST PROCEDURE

The number of organisms in the test culture prepared the previous day was estimated from the preliminary plates of that day, and this culture was

*Actual size. Photographed by Mrs. Miriam Wallace.

diluted on the day of the test so as to contain 100,000 to 600,000 cells per cubic centimeter. Appropriate dilutions of the antibacterial substance were distributed in tubes in 1 c.c. amounts, and 0.05 c.c. of the test culture was added to each tube. Two loopfuls of the control tube were at once spread out separately on two halves of an agar plate to determine the initial count. The test preparations were then placed in a water bath at 37° C. At one-hour, two-hour, or longer intervals, one loopful from each was spread over the surface of the agar allotted to it. Six antibacterial agents, each in eight dilutions, were easily tested at one-hour or two-hour intervals by one worker.

Where the antibacterial agent was dissolved in a solvent such as alcohol or carbowax,[1, 3] controls were made to determine whether the solvent itself affected the bacteria.

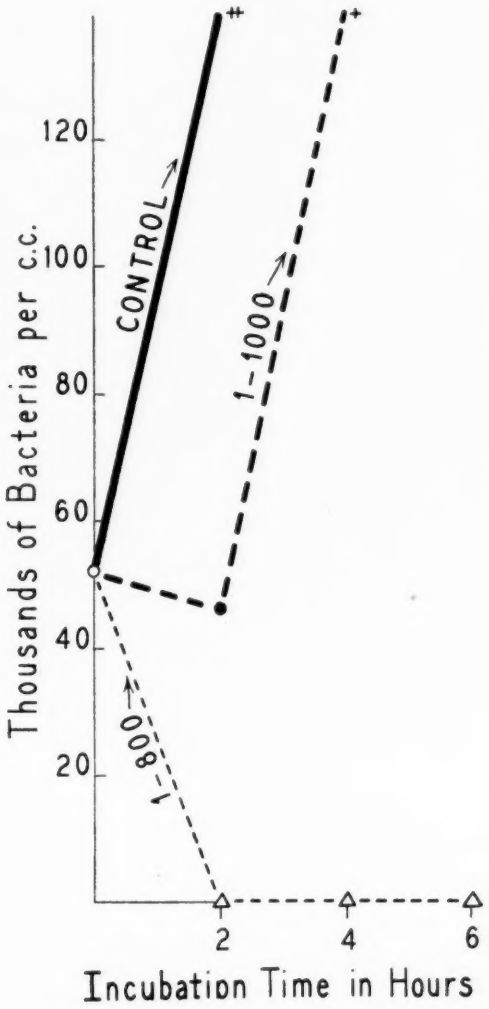


Fig. 1.—Antibacterial action of phenol for *Staph. aureus*.

RESULTS

Figs. 1 to 11* show the growth curves of *Staph. aureus* in the presence of the following antibacterial substances: phenol, streptothricin, streptomycin, clavacin, penicillin, protoanemonin, Manganese protoporphyrin, alcohol-sulfur, carbowax-sulfur, sulfanilamide, and pentathionic acid. Figs. 1 to 11 show single experiments, all of which have been repeated at least once with essentially similar growth curves.

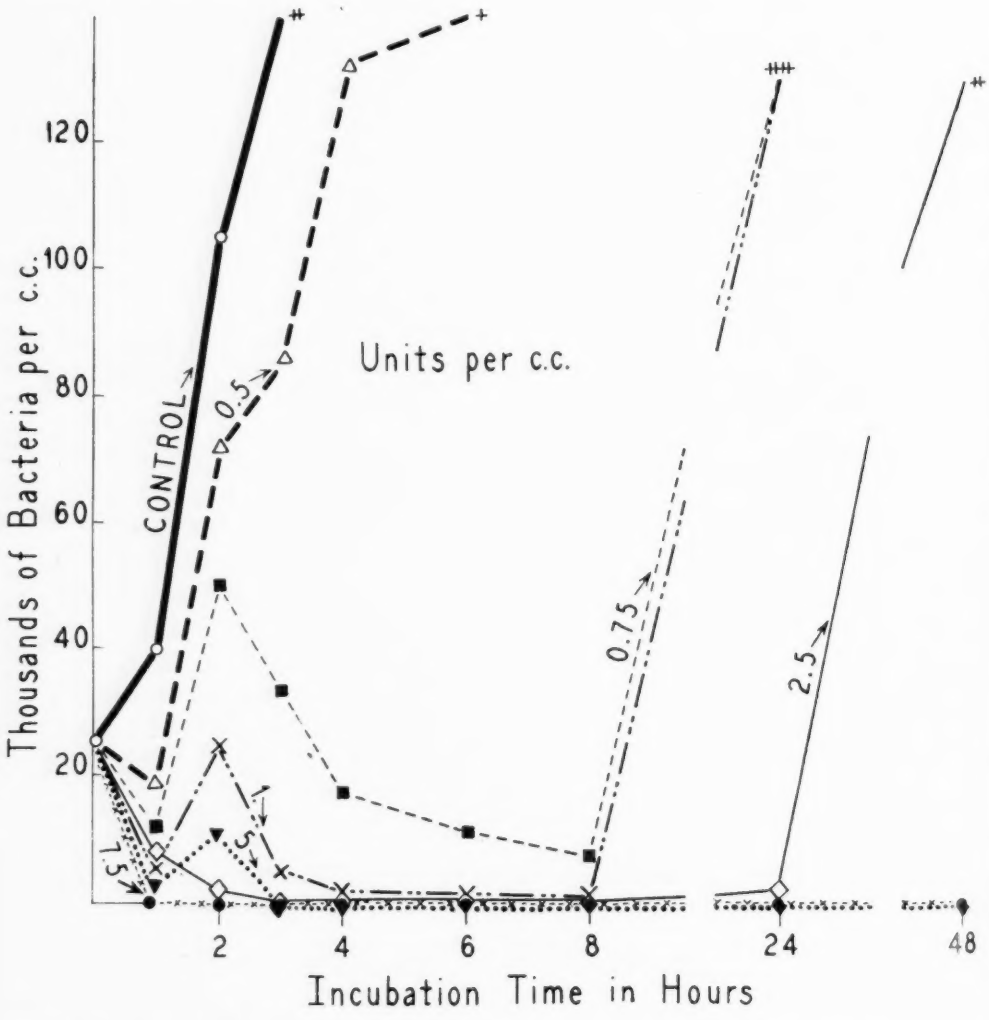


Fig. 2.—Antibacterial action of streptothricin for *Staph. aureus* (units per cubic centimeter).

Protoanemonin is an antibacterial agent, described by Seegal and Holden[4] and by Baer, Holden, and Seegal,[5] active not only against the growth of certain gram-positive and gram-negative bacteria, but also against *Cryptococcus hominis*

*We are indebted to Miss Natalie Pearlstein for the preparation of Figs. 1 to 11.

and *Candida albicans*. The preparation used was a distillate of the stems, leaves, and blossoms of *Anemone pulsatilla*. Mn. protoporphyrin has been shown by Granick and Gilder[6] to inhibit a variety of organisms including *Staph. aureus* and the acid-fast bacilli in concentrations of 0.5 to 3 gamma per cubic centimeter. The sulfur preparations were suspensions of sulfur in alcohol

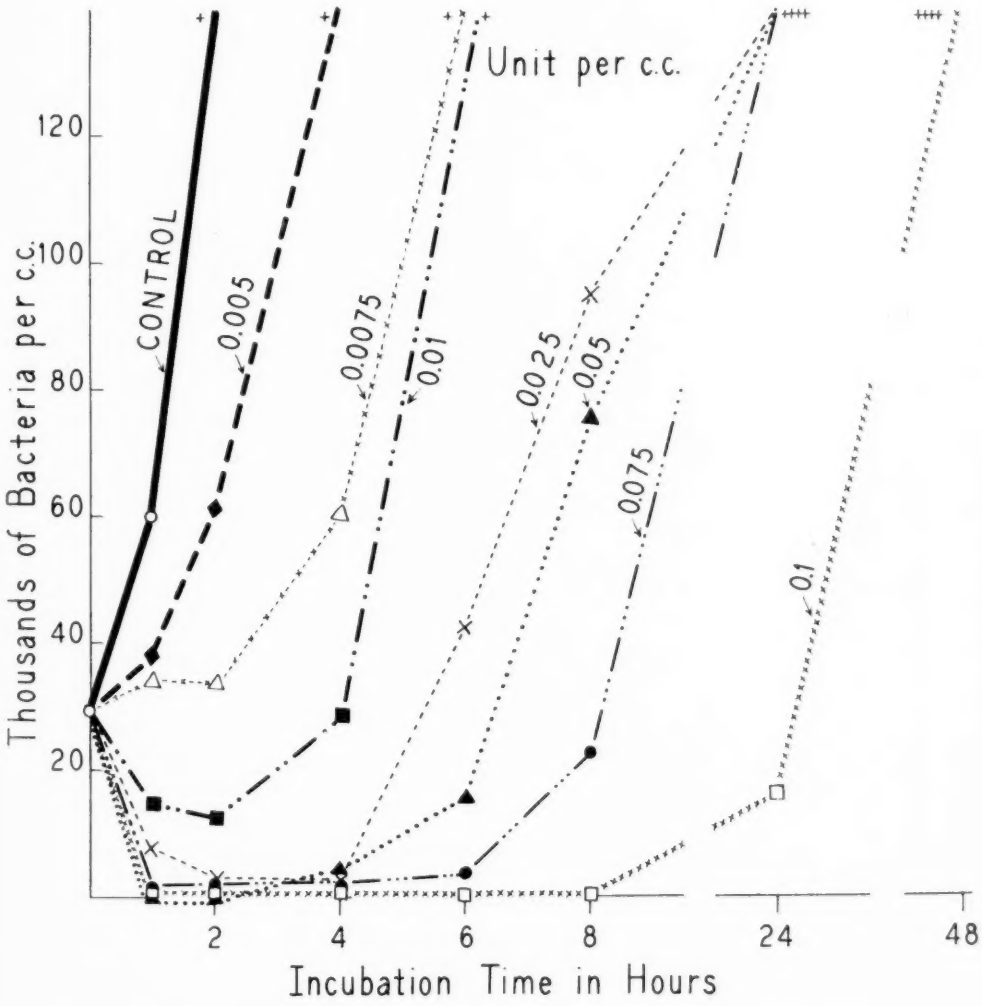


Fig. 3.—Antibacterial action of streptomycin for *Staph. aureus* (units per cubic centimeter).

(alcohol-sulfur) and in carbowax (carbowax-sulfur) which the authors have found inhibitory for *Staph. aureus* in preparations containing only 0.1 to 0.3 gamma of sulfur per cubic centimeter and for certain fungi in concentrations as low as 0.4 gamma. Pentathionic acid* was tested because it was suspected

*We are indebted to Dr. W. A. Knapp of the General Chemical Co., New York, N. Y., for making this material available to us. The $H_2S_5O_6$ prepared according to the Liming[7] method was an 8 per cent solution of $H_2S_5O_6$ containing tetra and trithionic acids but no thiosulfates.

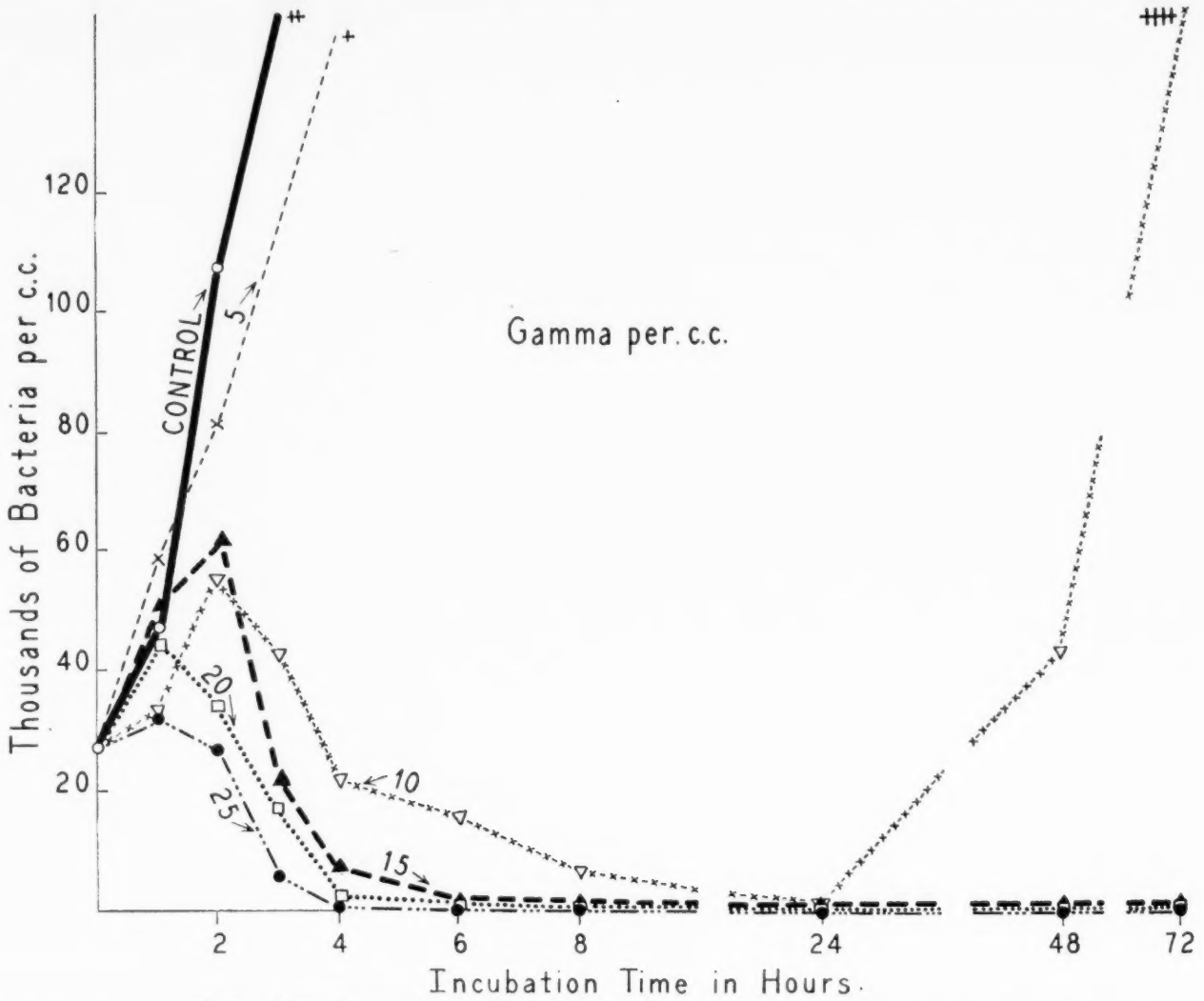


Fig. 1.—Antibacterial action of clavacin for *Staph. aureus* (gamma per cubic centimeter).

that if present in the sulfur preparations it might explain their extraordinary activity.

The curves showed at once important differences in the effects of these antibacterial agents in the ranges of concentration in which they were active. The more important differences are summarized in Table I. Even more interesting were the patterns of these curves obtained in the first eight hours of

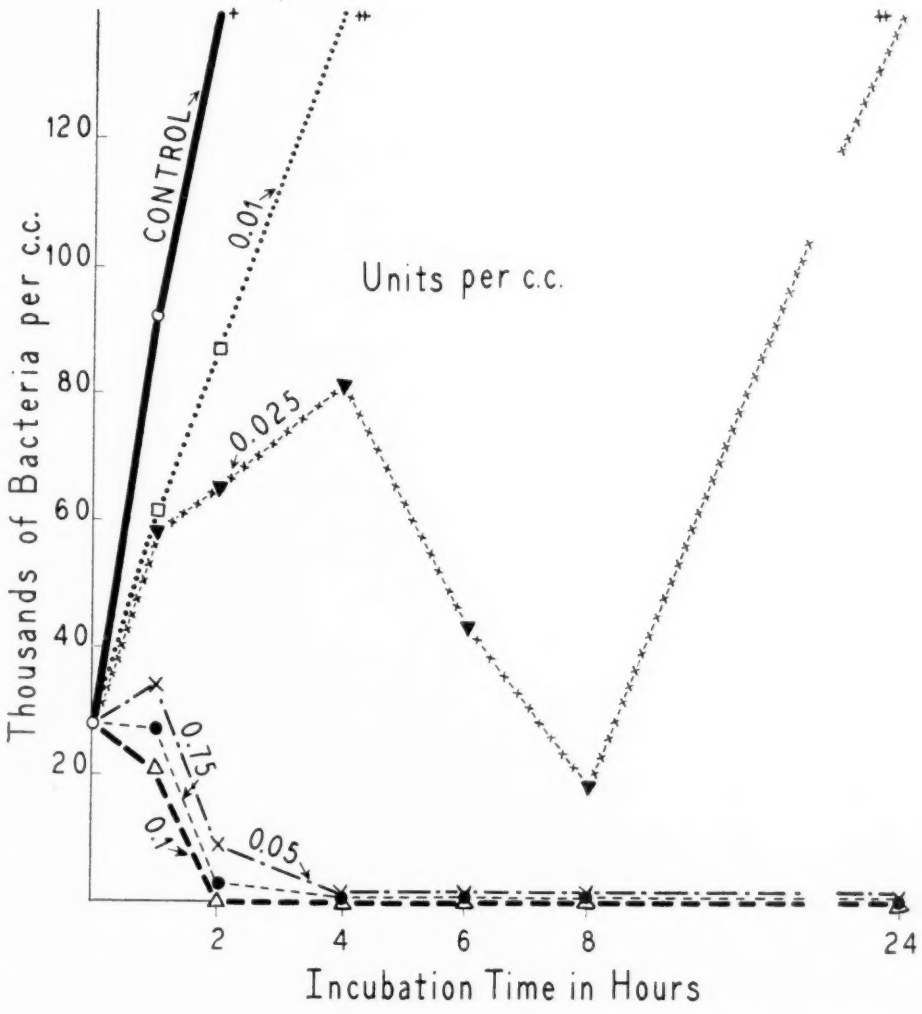


Fig. 5.—Antibacterial action of penicillin for *Staph. aureus* (units per cubic centimeter).

growth. These differed in each instance and seemed characteristic for the substances studied. The following phenomena were noted:

1. *Early Bactericidal Effect.*—The statement that certain agents were bactericidal is made with the same reservations necessary in testing for bactericidal effect by most methods in current use. It was not proved that the effect was

TABLE I. COMPARISON OF BACTERICIDAL AND BACTERIOSTATIC EFFECTS OF VARIOUS ANTIBACTERIAL AGENTS

| 1<br>ANTIBACTERIAL AGENT | 2<br>MINIMUM BACTERICIDAL DOSE | 3<br>BACTERICIDAL EFFECT IN HOURS | 4<br>MAXIMUM NONINHIBITING DOSE‡ | 5<br>RATIO $\frac{\text{COLUMN 2}}{\text{COLUMN 4}}$ |
|---|---|---|---|---|
| Group 1 | | | | |
| Phenol | 1-800* | 2 | 1-1,000 | 1.25 |
| Streptothricin | 5 u./c.c.* | 3 | 0.5u./c.c. | 10 |
| Streptomycin | 0.2 u./c.c.* | 1 | 0.005 | 40 |
| Clavacin | 25 μg c.c.* | 6 | 5 μg/c.c. | 5 |
| Penicillin | 0.05 u./c.c.* | 4 | 0.01 u./c.c. | 5 |
| Protoanemonin | 1-20,000* | 8 | 1-60,000 | 3 |
| Group 2 | 10 μg/c.c.† | – | 0.125 μg/ c.c. | 80 |
| Mn. Protoporphyrin | 1-100† | – | 1-51,200 | 512 |
| Alcohol-sulfur | 1-100† | – | 1-800,000 | 8,000 |
| Carbowax-sulfur | | | | |

*No growth from one loop (0.002 c.c.) inoculum.

†No bactericidal effect in largest amounts tested in eight hours.

‡Maximum dose of antibacterial agent giving curve approximately like that of control. Doses listed delayed growth slightly. See Fig. 2, 0.5 unit curve; Fig. 3, 0.005 unit curve; Fig. 4, 5 μg curve, etc.

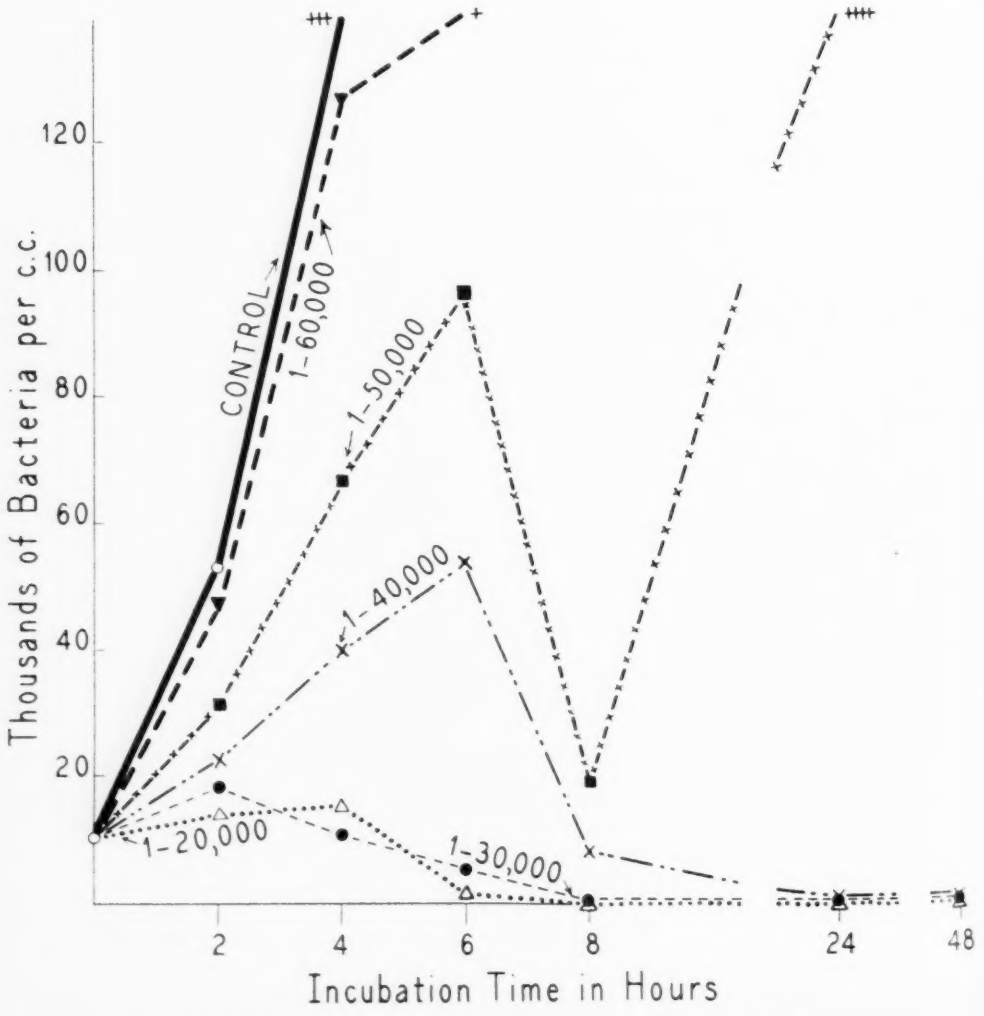


Fig. 6.—Antibacterial action of protoanemonin for *Staph. aureus*.

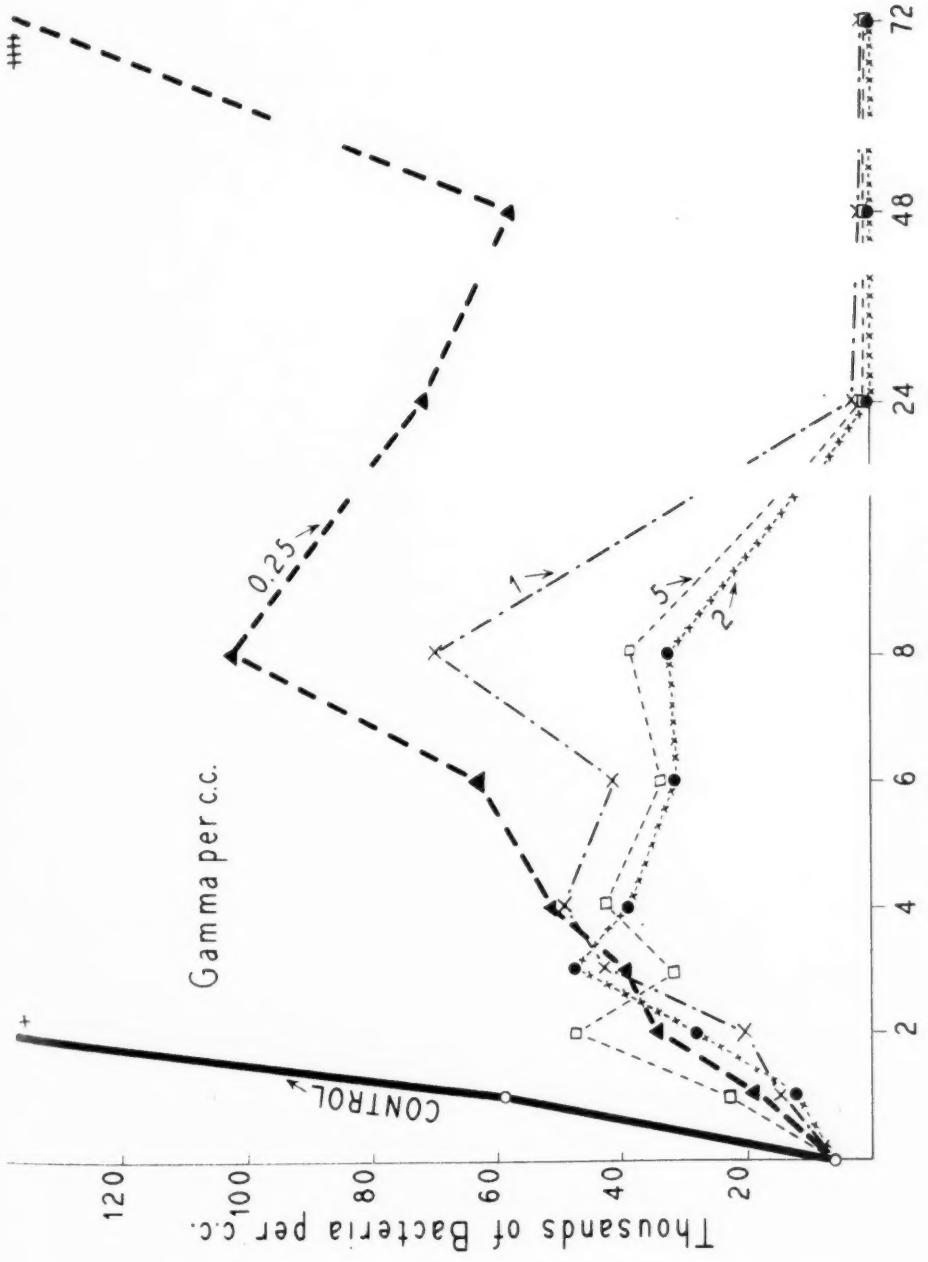


Fig. 7.—Antibacterial action of Mn. protoporphyrin for *Staph. aureus* (gamma per cubic centimeter).

irreversible, nor was it proved that subculture of a larger sample might not have shown a few surviving cells.

It was evident, however, that the effect of phenol was purely bactericidal in the usually accepted sense. High concentrations of streptothricin, streptomycin,* and penicillin also appeared bactericidal within two hours. A slower

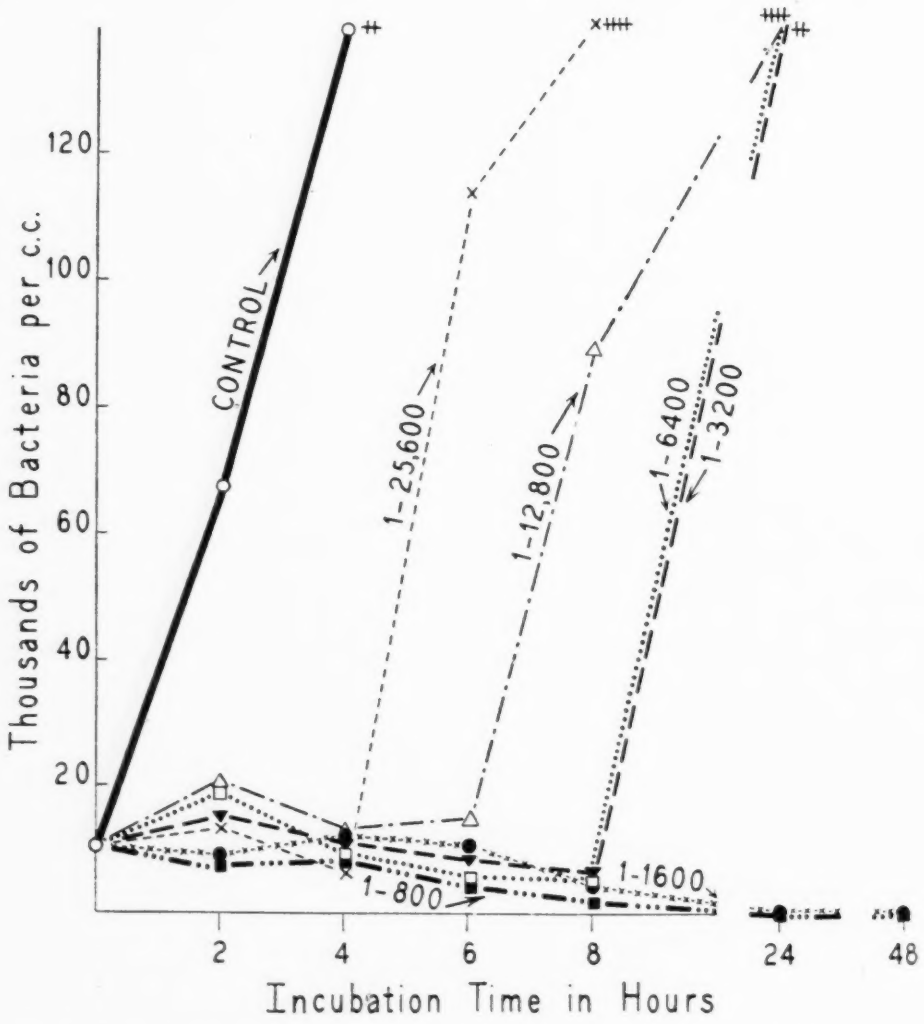


Fig. 8.—Antibacterial action of alcohol solution of sulfur (alcohol-sulfur) for *Staph. aureus*.

bactericidal effect is shown by clavacin and protoanemonin in six and eight hours, respectively. The other three antibacterial agents (Mn. protoporphyrin, alcohol-sulfur, and carbowax-sulfur), even in the highest concentrations used, showed no bactericidal effect during the first eight-hour period of growth.

*The 0.2 unit curve which gave negative cultures at one, two, four, six, eight, twenty-four, and forty-eight hours is omitted from Fig. 3.

Sulfanilamide also showed no bactericidal effect, but this may have been due to its insolubility in sufficiently high concentration. Adequate test of pentathionic acid could not be made because high concentrations were too acid to permit growth of the staphylococcus.

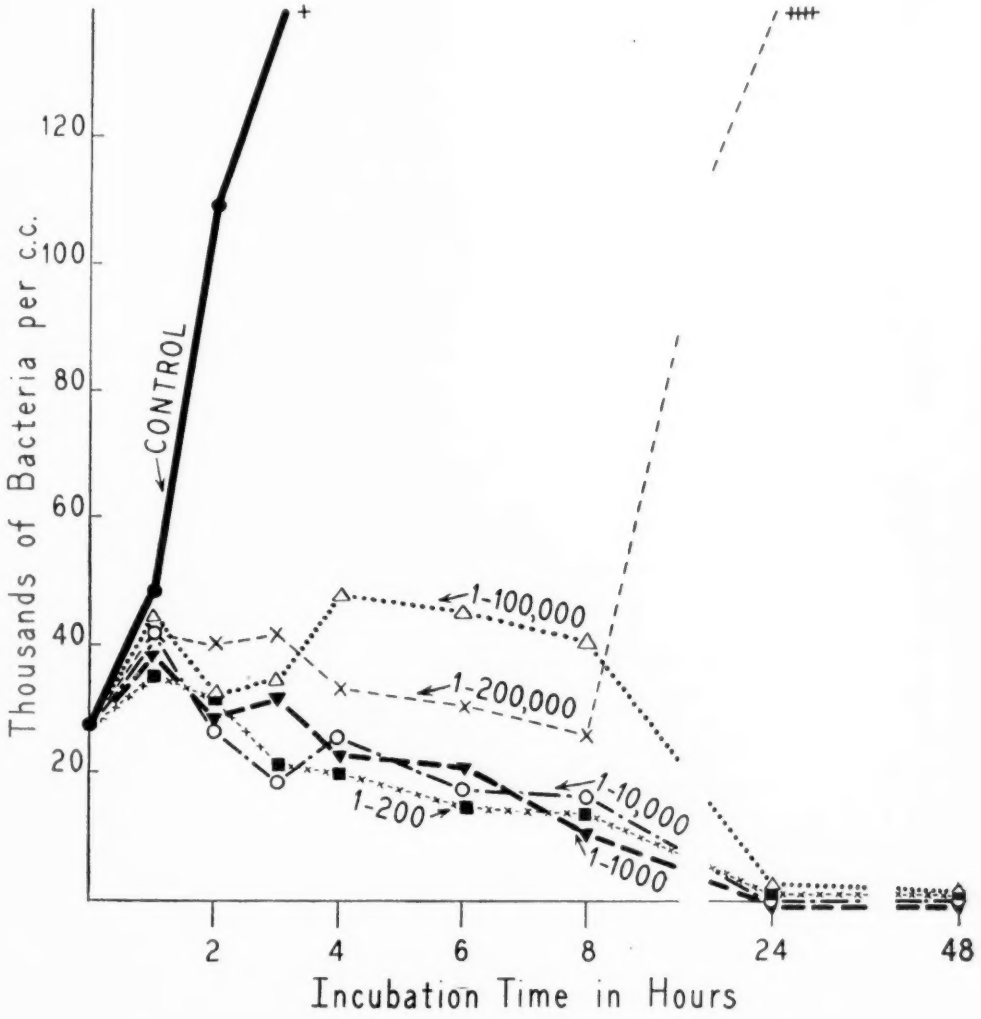


Fig. 9.—Antibacterial action of carbowax solution of sulfur (carbowax-sulfur) for *Staph. aureus.*

2. *The Phase of Early Growth Followed by Inhibition.*—This occurs with intermediate concentrations of several of the antibacterial agents tested. It is seen at approximately two hours with streptothricin. A more prolonged growth phase with a peak of growth at six hours is shown by protoanemonin and sulfanilamide. Mn. protoporphyrin differs from streptothricin and protoanemonin in that it permitted rapid early growth with peaks at four to eight hours. The other antibacterial agents tested show no definite early phase of growth.

3. *Bacteriostatic Effect.*—All agents tested showed a zone in which the cocci were inhibited for a period but began active growth within eight hours. With phenol the period lasted only two hours in a narrow zone of concentration. With protoanemonin, clavacin, and penicillin the range of concentration showing only inhibition was also narrow. It was somewhat wider in streptothricin, streptomycin, and Mn. protoporphyrin. The curves for sulfanilamide also

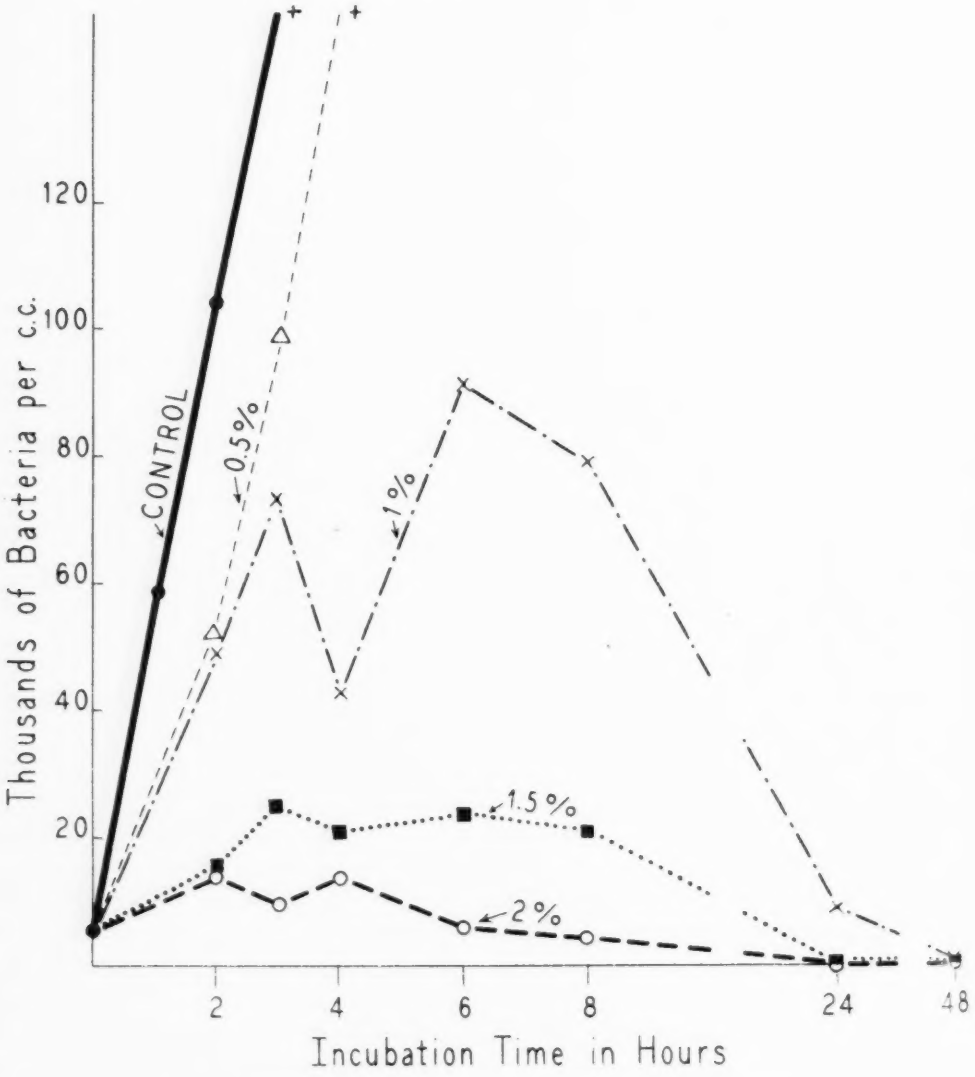


Fig. 10.—Antibacterial action of sulfanilamide for *Staph. aureus.*

showed inhibition only in a narrow zone, but this may have been due to the presence of sulfonamide antagonists in the medium which counteracted its effect except in high concentration. The low pH of strong solutions of pentathionic acid also prevented determination of its range of activity.

With the sulfur suspensions, inhibition seemed of a different order. In these there was an enormous range of concentrations in which the organisms were held at about the inoculation level for from four to eight hours, only to grow out freely at a later period. Tubes with higher concentrations in which the organisms were inhibited for twenty-four hours or longer appeared sterile on transfer. It seemed more probable that this was due to senescence of the inhibited organisms rather than to direct bactericidal effect.[5]

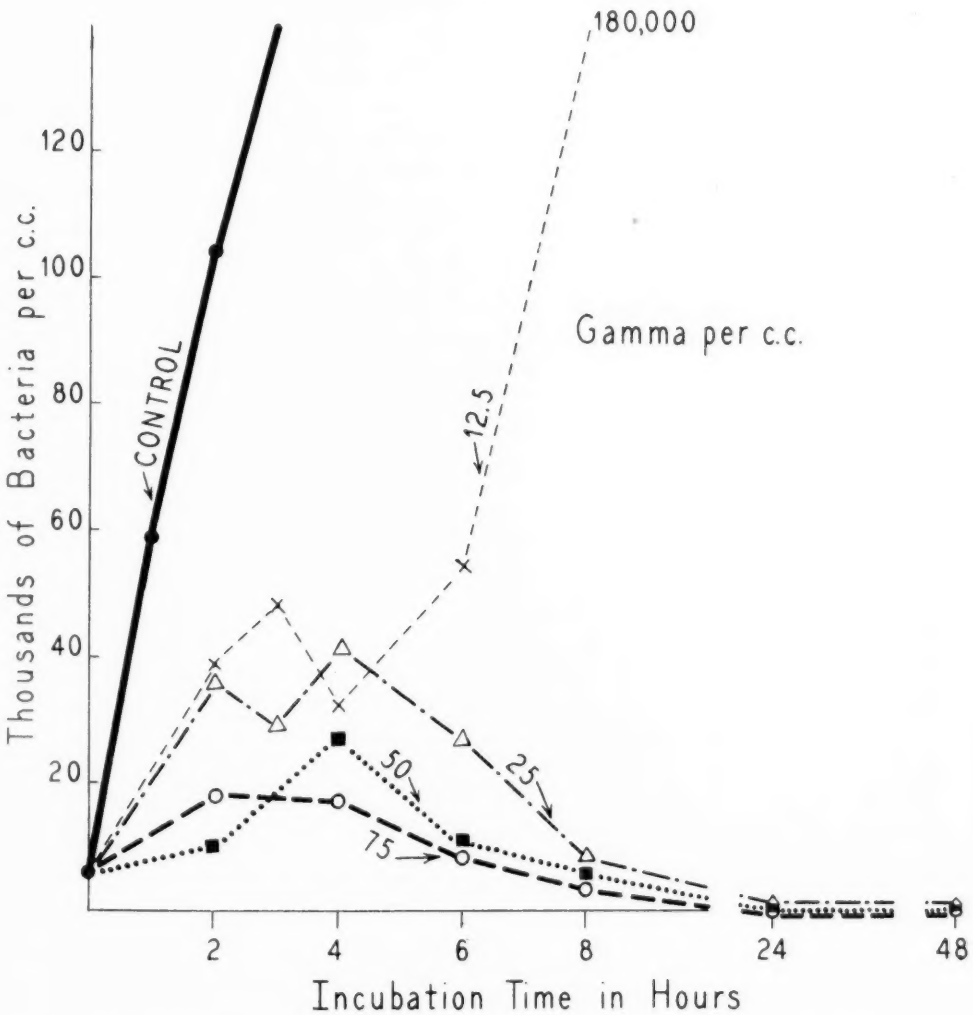


Fig. 11.—Antibacterial action of pentathionic acid for *Staph. aureus* (gamma per cubic centimeter).

4. *The Renewal of Growth Phenomenon.*—This was seen after eight to twenty-four hours' growth, with intermediate concentrations of several antibacterial substances after the organisms had practically disappeared from the cultures and appeared to be dying out. This was probably due to the presence

of a small number of resistant organisms in the test culture.[9-11] This phenomenon is most apparent with streptothricin and streptomycin and, to a less extent, with clavacin and possibly with Mn. protoporphyrin. It was not seen in the sulfur preparations or with sulfanilamide. In concentrations of these latter agents, which inhibited the staphylococci for eight hours, the organisms failed to grow out after longer exposure.

Table I demonstrates that the substances tested may be separated roughly into two groups:

1. Antibacterial agents which are bactericidal in eight hours or less. In this group the minimum bactericidal dose is only 40 or less times the maximum noninhibiting dose. The range of dilutions which gives inhibition without bactericidal effect is small.

2. Antibacterial agents which are merely bacteriostatic in eight hours. This group gives only bacteriostasis in doses as high as 80 to 8,000 times the maximum noninhibiting dose. The range of dilutions which gives inhibition is large.

## SUMMARY

A simple streak plate method for demonstrating growth curves of *Staph. aureus* under the influence of antibacterial agents is described.

Graphs are presented showing the effect of the following antibacterial agents:

*Phenol:* A rapidly bactericidal substance which kills the organisms in a concentration only slightly higher than one which has even no inhibiting effect.

*Streptothricin, Streptomycin, Clavacin, Penicillin and Protoanemonin:* Antibacterial agents which kill the organisms in eight hours or less in concentrations not more than 40 times the noninhibiting doses.

*Mn. protoporphyrin and Suspensions of Sulfur in Alcohol (Alcohol-Sulfur) and in Carbowax (Carbowax-Sulfur):* Purely bacteriostatic preparations which fail to kill the organisms in eight hours in the highest concentrations tested but inhibit growth completely in minute fractions of such amounts.

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# FLUID THIOGLYCOLLATE MEDIUM

## Its Use for Testing the Sterility of Surgical Catgut Sutures

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Brewer,[1] in 1940, announced his fluid thioglycollate medium for the cultivation of anaerobes and stated that it also would support growth of aerobes. In his table, he showed that *Clostridium novyi* would grow satisfactorily up to a month after sterilization of the culture medium, but he did not mention the size of the inoculum used. He stated in his paper: "The sodium thioglycollate combines with and inactivates most of the mercurials used as preservatives, and one is much more likely to obtain growth from contaminated biologicals which are preserved with one of these highly bacteriostatic compounds."

McClung[4] reported that Brewer's thioglycollate medium compares favorably with complex meat infusions in its ability to initiate growth from small numbers of cells. For his tests, eleven different species of anaerobes including *Cl. novyi* were used.

Nungester, Hood, and Warren[6] conducted experiments to determine whether the addition of sodium thioglycollate to culture medium used in testing disinfectants by an in vitro method would give results similar to those obtained by the "infection-prevention" method. They experimented with six mercurial compounds and found the percentage of positive growths was much greater when a culture medium containing thioglycollate was used. They concluded: "Sodium thioglycollate added to media for testing disinfectants *in vitro* apparently neutralized the bacteriostatic effect of the mercurial compounds tested and thus indicated the inadequacy of these substances as germicides."

As a result of Brewer's findings, that the addition of sodium thioglycollate to culture medium inhibits the bacteriostatic action of mercurials, the National Institute of Health[5] specified a formula for fluid thioglycollate medium which it recommends for use in testing the sterility of biologic products. This culture medium* is a modification of the original Brewer formula.

It has been suggested that fluid thioglycollate medium might be used effectively to test the sterility of all products that are preserved or impregnated with mercurial compounds. Such a procedure would utilize the fluid thioglycollate medium for testing the sterility of surgical catgut sutures instead of the culture media officially prescribed by the *United States Pharmacopoeia XII*.

### PURPOSE OF INVESTIGATION

The purpose of this investigation was twofold: (1) to determine the comparative growth-promoting properties of the National Institute of Health formula for fluid thioglycollate medium with those of the Novy culture medium which is specified in the U. S. P. XII as the official anaerobic medium, and (2)

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*Prepared by the Difco Laboratories, Incorporated, Detroit, Mich.

to determine the efficiency of the fluid thioglycollate medium for testing the sterility of surgical catgut sutures *without* employing the inactivating fluids which constitute part of the U. S. P. XII Sterility Tests for Solids.

## MATERIALS AND METHODS

The culture media comprised (1) bacto-fluid thioglycollate medium and (2) the U. S. P. XII medium prepared for aerobiosis and that prepared for anaerobiosis. The fluid thioglycollate medium was incubated without seal, while tubes of the U. S. P. XII anaerobic medium were sealed with a mixture of equal parts of petroleum jelly and paraffin. The final pH of the fluid thioglycollate medium was adjusted to 7.1 as recommended by Pittman,[7] while that of the U. S. P. XII media was adjusted to 7.4 as specified in the U. S. P. XII.

A series of experiments was conducted to determine the growth-promoting properties of the fluid thioglycollate medium and of the U. S. P. XII anaerobic medium, using *Cl. novyi* as the test organism in decimal dilutions. In the official Sterility Tests for Solids, the U. S. P. XII specifies[8] *Cl. novyi* for the control organism to determine the growth-promoting properties of the culture medium.

Another series of experiments involved the testing for sterility of catgut sutures artificially infected with a sporulating culture of *Clostridium sporogenes* and *Bacillus subtilis*, and impregnated with mercuric iodide to the amount of 1 per cent in some of the sutures and 3.5 per cent in others.

## EXPERIMENTS TO DETERMINE GROWTH-PROMOTING PROPERTIES

Several tubes of the fluid thioglycollate medium and of the U. S. P. XII anaerobic medium were inoculated with 1 c.c. of decimal dilutions (from $10^{-1}$ to $10^{-10}$) of a forty-eight hour broth culture of *Cl. novyi*. Typical protocols are shown in Table I. This experiment was repeated several times and the results were similar to those shown in the table.

*Discussion of Results.*—

1. *Fluid Thioglycollate Medium:* After twenty-four hours gas formed in the first dilution, and a very light bacterial growth occurred from an inoculum of 0.000001 c.c. ($10^{-6}$).

After forty-eight hours gas formed in the first five tubes.

After five days maximum degree of growth (4 plus) occurred from an inoculum of 0.00001 c.c. ($10^{-5}$), gas formed from an inoculum of 0.000001 c.c. ($10^{-6}$), and a very light bacterial growth was present in the tube containing an inoculum of 0.00000001 c.c. ($10^{-8}$).

2. *U. S. P. XII Anaerobic Medium:* After twenty-four hours, a very light bacterial growth was present in the tube containing an inoculum of 0.00001 c.c. ($10^{-5}$), but gas did not form in any of the tubes.

After forty-eight hours gas formed in the first four tubes.

After five days maximum degree of growth (4 plus) occurred from an inoculum of 0.001 c.c. ($10^{-3}$), gas formed from an inoculum of 0.00001 c.c. ($10^{-5}$), and a light bacterial growth occurred from an inoculum of 0.000001 c.c. ($10^{-6}$).

TABLE I

| MEDIUM | INCUBATION PERIOD | DECIMAL DILUTIONS | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ | $10^{-7}$ | $10^{-8}$ | $10^{-9}$ | $10^{-10}$ |
| Bacto-fluid thioglycollate medium | 24 hr. | 4+ gas | 3+ | 3+ | 2+ | 2+ | + | 0 | 0 | 0 | 0 |
| | 48 hr. | 4+ gas | 4+ gas | 4+ gas | 3+ gas | 3+ gas | 2+ | + | 0 | 0 | 0 |
| | 5 days | 4+ gas | 4+ gas | 4+ gas | 4+ gas | 4+ gas | 3+ gas | 2+ | + | 0 | 0 |
| U. S. P. medium for anaerobiosis | 24 hr. | 4+ | 3+ | 2+ | + | + | 0 | 0 | 0 | 0 | 0 |
| | 48 hr. | 4+ gas | 4+ gas | 3+ gas | 3+ gas | 2+ | + | 0 | 0 | 0 | 0 |
| | 5 days | 4+ gas | 4+ gas | 4+ gas | 3+ gas | 3+ gas | 2+ | 0 | 0 | 0 | 0 |

The degree of bacterial growth is indicated by plus signs: very light cloud, 1 plus; light cloud, 2 plus; heavy cloud, 3 plus; and very heavy cloud, 4 plus. Absence of growth is indicated by 0.

Decimal dilutions of a forty-eight hour broth culture of *Cl. novyi* were used to determine the comparative growth-promoting properties of bacto-fluid thioglycollate medium and the U. S. P. XII anaerobic medium. In the tubes of bacto-fluid thioglycollate medium, gas formed earlier and after five days was present in one higher dilution than in the tubes of the U. S. P. anaerobic medium. Bacterial growth was present on the fifth day in the tubes of bacto-fluid thioglycollate medium as far out as the eighth dilution in contrast to the sixth dilution in the tubes of the U. S. P. anaerobic medium.

These results indicate that the fluid thioglycollate medium is superior to the U. S. P. XII anaerobic medium for supporting the growth of anaerobic bacteria, such as *Cl. novyi*. This superiority is shown (1) after twenty-four hours' incubation, by gas formation in the first dilution and by bacterial growth out as far as the sixth dilution; (2) after forty-eight hours' incubation, by the maximum degree of growth occurring in one higher dilution and by bacterial growth being present in one higher dilution; and (3) after five days' incubation, by gas formation in one higher dilution, by the maximum degree of bacterial growth occurring in two dilutions higher, and by bacterial growth being present out as far as the eighth dilution in contrast to the sixth dilution.

## EXPERIMENTS ON INACTIVATION OF MERCURIALS

To determine the extent of the combining and inactivating properties of thioglycollic acid (contained in the fluid thioglycollate medium) for mercury, a series of experiments was carried out in which artificially infected catgut sutures containing 1 per cent mercuric iodide (Lot 1754) and some containing 3.5 per cent mercuric iodide (Lot 1755) were tested for sterility. The sutures were infected with a sporulating culture of *Clostridium sporogenes* and *Bacillus subtilis*. For controls, sutures artificially infected with the sporulating culture but not impregnated with any chemical compound (Lot 1753) were used.

Comparative tests were made by placing some of each of the three different lots of sutures directly into tubes containing 40 c.c. fluid thioglycollate medium and incubating at 37° C.; other sutures of each of the three lots were tested in accordance with the sterility technique prescribed by the U. S. P. XII. This latter technique consisted of incubating the sutures first in tubes of 40 c.c. distilled water, then in tubes containing the inactivating fluid of 1 per cent

sodium thiosulfate and 1 per cent sodium carbonate; on the third day the sutures were transferred to tubes of distilled water and incubated twenty-four hours; and on the fourth day the sutures were transferred to tubes of beef broth prepared for aerobiosis and incubated, or transferred to tubes of gelatin beef broth prepared for anaerobiosis and sealed with a mixture of petroleum jelly and paraffin and then incubated. Results of these comparative tests are shown in Tables II and III.

TABLE II

| LOT | BACTO-FLUID THIOGLYCOLLATE MEDIUM | |
|---|---|---|
| | AEROBIC RESULTS | ANAEROBIC RESULTS |
| 1753 (Control) | 24-hour growth, gram-positive rods | 24-hour growth: gram-positive rods with subterminal spores; gas formation |
| 1754 (Containing 1% mercury) | 24-hour growth, gram-positive rods | 24-hour growth: gram-positive rods with subterminal spores; gas formation |
| 1755 (Containing 3.5% mercury) | 24-hour growth, gram-positive rods | 24-hour growth: gram-positive rods with subterminal spores; gas formation |

The inactivating properties of bacto-fluid thioglycollate medium for mercury were determined by sterility tests of catgut sutures artificially infected with a sporulating culture. Some sutures contained 1 per cent mercury, while others were impregnated with 3.5 per cent mercury. For controls, sutures artificially infected but not impregnated with mercury were used. These tests show that the thioglycollic acid in the bacto-fluid thioglycollate medium combines with and inactivates 1 per cent mercury and 3.5 per cent mercury, so that twenty-four hour growths occurred in all tubes and corresponded to the growths in the control tubes.

The results given in Table II indicate that the thioglycollic acid in the fluid thioglycollate medium combines with and inactivates the 1 per cent of mercury and the 3.5 per cent of mercury, as shown by the aerobic and anaerobic bacterial growths which occurred in Lots 1754 and 1755 and which checked with the control Lot 1753.

Table III shows the results of using the U. S. P. XII technique. It will be noted that the mercurial salts present in the sutures to the amount of 1 per cent combine with the sodium thiosulfate of the inactivating fluid to form a double salt which is soluble in water, as shown by the aerobic and anaerobic bacterial growths in Lot 1754. However, the amount of mercury in sutures of Lot 1755 is so great (3.5 per cent) that all of it will not combine with the sodium thiosulfate of the inactivating fluid, and enough mercury is still present in the sutures to exert a bacteriostatic effect and prevent bacterial growth.

TABLE III

| LOT | U.S.P. AEROBIC MEDIUM | U.S.P. ANAEROBIC MEDIUM |
|---|---|---|
| | AEROBIC RESULTS | ANAEROBIC RESULTS |
| 1753 (Control) | 24-hour growth, gram-positive rods | 24-hour growth: gram-positive rods with subterminal spores; gas formation |
| 1754 (Containing 1% mercury) | 24-hour growth, gram-positive rods | 24-hour growth: gram-positive rods with subterminal spores; gas formation |
| 1755 (Containing 3.5% mercury) | No growth after 15 days' incubation | No growth after 15 days' incubation |

Experiments were made to determine the inactivating properties for mercury of the standard inactivating fluid of 1 per cent sodium thiosulfate and 1 per cent sodium carbonate prescribed by the U. S. P. XII. Catgut sutures artificially infected and containing 1 per cent mercury and some containing 3.5 per cent mercury were tested for sterility by the U. S. P. XII technique. For controls, sutures artificially infected but not impregnated with mercury were used. These tests show that the standard inactivating fluid combines with and inactivates 1 per cent mercury (as shown by twenty-four hour growths in Lot 1754), but that it is not capable of combining with and inactivating 3.5 per cent mercury (as shown by the absence of growth in Lot 1755).

In my bacteriologic investigations of chemical sterilization of surgical catgut sutures, I devised[2] an inactivating fluid of 10 per cent sodium thiosulfate for effectively combining with and inactivating mercurial compounds if present in amounts greater than 2 per cent. I recommended[3] the use of this 10 per cent sodium thiosulfate solution as an inactivating fluid whenever catgut sutures are found upon chemical analyses to contain more than 2 per cent of mercury. The U. S. P. XII adopted[8] this procedure as part of the official Sterility Tests for Solids.

Accordingly, additional sutures from Lot 1755 were tested by the U. S. P. XII technique in which the 10 per cent sodium thiosulfate inactivating fluid was used, with results shown in Table IV.

TABLE IV

| LOT | U.S.P. AEROBIC MEDIUM | U.S.P. ANAEROBIC MEDIUM |
|---|---|---|
| | AEROBIC RESULTS | ANAEROBIC RESULTS |
| 1753 (Control) | Growth in 24 hours, gram-positive rods | Growth in 24 hours: gram-positive rods with subterminal spores; gas formation |
| 1755 (Containing 3.5% mercury) | Growth in 24 hours, gram-positive rods | Growth in 24 hours: gram-positive rods with subterminal spores; gas formation |

Experiments were made to determine the inactivating properties of the special inactivating fluid of 10 per cent sodium thiosulfate prescribed in the U. S. P. XII sterility technique when large amounts of mercury are present in sutures. Catgut sutures artificially infected with a sporulating culture and containing 3.5 per cent mercury were tested for sterility. Sutures artificially infected but not impregnated with mercury were used for controls. These tests show that the special inactivating fluid of 10 per cent sodium thiosulfate combines with and inactivates 3.5 per cent mercury, so that in all tubes of Lot 1755 growths occurred in twenty-four hours, which corresponded to growths in the controls.

These results demonstrate that the special inactivating fluid of 10 per cent sodium thiosulfate is required in connection with the U. S. P. XII technique in order to detect the presence of living bacteria in catgut sutures which are heavily impregnated with mercurial salts. This special inactivating fluid eliminates all bacteriostatic action of the mercury, thereby allowing the bacteria in the catgut to germinate in the culture medium.

*Discussion of Results.*—This series of experiments to determine the inactivation of mercurials demonstrates in a convincing manner that the fluid thioglycollate medium is superior to the U. S. P. XII culture media for testing the sterility of surgical catgut sutures. It simplifies the technique because it may be used *without* the inactivating fluids specified in the U. S. P. XII for the inactivation of mercurial salts. The thioglycollic acid contained in the fluid thioglycollate medium combines with the mercury and thereby eliminates all bacteriostatic action of mercurial salts when present in catgut sutures in amounts as great as 3.5 per cent.

## CONCLUSIONS

The experiments herein described demonstrate that bacto-fluid thioglycollate medium is superior, in the following respects, to the U. S. P. XII culture media for testing the sterility of surgical catgut sutures:

1. A smaller inoculum supports the growth of anaerobic bacteria, such as *Cl. novyi.*

2. Gas forms earlier, and maximum bacterial growth occurs from a much smaller inoculum.

3. No seal is required for anaerobiosis, since the sodium salt of thioglycollic acid lowers the oxidation reduction potential which is maintained for a considerable period of time by the agar in the medium.

4. Preliminary incubation in distilled water and inactivating fluids, for the purpose of removing bacteriostatic mercurial salts with which the sutures may be impregnated, is not required.

5. The sodium salt of thioglycollic acid in the culture medium effectively neutralizes the bacteriostatic action of mercurial compounds when present in catgut sutures in amounts up to 3.5 per cent.

Since these experiments were completed, the Committee of Revision of the United States Pharmacopoeia adopted the National Institute of Health formula for fluid thioglycollate medium for use in Sterility Tests for Liquids and Solids, and it will become the official sterility test medium in the U. S. P. XIII.

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# DETERMINATION OF INULIN IN PLASMA AND URINE

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THE use of inulin in measuring glomerular filtration has stimulated the development of a great number of methods for the estimation of this substance in plasma and urine. Among the reagents used, diphenylamine appears to yield the greatest color density per milligram of inulin present. However, even employing this reagent in Harrison's* modification of the method of Alving, Rubin, and Miller, we found that the color produced by low concentrations of inulin (4 to 20 mg. per cent) proved to be too feeble to permit exact readings in the Pulfrich photometer. Since it is known that the color intensity obtained with diphenylamine is greatly reduced in the presence of water, we attempted to find a method for precipitating inulin from the plasma filtrates and for subsequently carrying out the estimation with the dried precipitate. Such a method would have an additional advantage in that larger aliquots could be analyzed than have been possible with current methods.

In preliminary experiments it was found that inulin can be quantitatively precipitated from solution by treatment with calcium hydroxide. No sugar can be detected in the supernatant fluid, nor is any sugar eluated by subsequent washing of the precipitate with alkaline water or with alcohol. On addition of the strongly acid diphenylamine reagent, calcium hydroxide is easily dissolved, setting free the precipitated inulin for hydrolysis and color production.

The following method was evolved for the determination of inulin in blood and urine as part of the measurement of inulin clearance in man.

## METHOD

*Solutions.*—

Zinc sulfate solution 10 per cent.

Sodium hydroxide solution N/2.

Calcium chloride solution 2N.

Sodium hydroxide solution 2N.

Saturated solution of calcium hydroxide in water.

Saturated solution of calcium hydroxide in alcohol.

Diphenylamine reagent. 3 Gm. diphenylamine dissolved in 100 ml. glacial acetic acid. Add 60 ml. concentrated hydrochloric acid. Store in amber bottle in a cold place.

Inulin stock solution. 100 mg. inulin in 100 ml. water containing 1 drop of N/10 sodium hydroxide. This solution keeps for two weeks in a cold place.

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*Harrison, H. E.: A Modification of the Diphenylamine Method for Determination of Inulin, Proc. Soc. Exper. Biol. & Med. 49: 111, 1942.

Working standard. Freshly prepared from the stock solution by diluting 2 to 100 ml. with water.

*Procedure.*—Measure 0.2 to 0.5 ml. of plasma or serum containing 10 to 100 μg of inulin into a centrifuge tube. Make up to 9 ml. with water, add 0.5 ml. zinc sulfate solution, and mix. Add 0.5 ml. of N/2 sodium hydroxide solution, mix, centrifuge, and filter. Measure 5 ml. of the clear filtrate into a centrifuge tube and add 0.5 ml. calcium chloride solution and 0.5 ml. 2N sodium hydroxide solution. Heat in a boiling water bath for five minutes. Centrifuge sharply for five minutes. Decant the supernatant fluid. Wash the precipitate with 5 ml. of the aqueous solution of calcium hydroxide, using a small glass rod for each tube in order to break up the larger clumps. Put the rods aside for further use with their respective tubes. Centrifuge the suspension sharply, decant, and wash with alcohol-calcium hydroxide as described. After decantation drive off the remaining alcohol by immersing the tubes in a boiling water bath for five minutes. Return the rods to their tubes. Add 5 ml. of diphenylamine reagent, stir well, and heat in a boiling water bath for exactly thirty minutes. Cool in cold water. Read in a photometer with a filter transmitting at 610 millimicrons.

Each run includes one plasma blank from the patient's blood before the injection, and two standards, prepared by adding 0.5 ml. of the blank plasma to 2 ml. and 4 ml. of the standard inulin solution, making up to 9 ml., and proceeding as described.

Urine is diluted 500- to 1,000-fold. To 5 ml. of the diluted urine add 0.5 ml. of calcium chloride solution, and proceed as with the blood filtrates. A blank value is determined from the patient's urine before the test.

*Calculation.*—The extinction values obtained with the two standards included in the series give the factors needed for calculation of both plasma concentration and urinary excretion.

## DISCUSSION OF METHOD

*Color Intensity.*—With this method, 10 μg of inulin in the final solution gave an extinction of 0.2 with a 10 mm. cell. This value was practically constant. We do not, however, propose to dispense with the inclusion of standards in each run, since changes might occur in heating conditions or in various reagent batches which would otherwise remain undetected.

Our method achieves color intensities five times as strong as those obtained with Harrison's method (Table I). This is accounted for by our use of 5 ml. of

TABLE I. COMPARISON OF METHODS

| INULIN IN PLASMA (MG. %) | EXTINCTION IN 1 CM. CELL MINUS BLANK | |
|---|---|---|
| | ACCORDING TO HARRISON'S METHOD | ACCORDING TO OUR METHOD |
| 4 | 0.04 | 0.20 |
| 8 | 0.08 | 0.40 |
| 20 | 0.18 | 1.04 |

TABLE II. RECOVERY OF INULIN ADDED TO PLASMA

| PLASMA (ML.) | INULIN ADDED (MG. %) | INULIN RECOVERED (MG. %) |
|---|---|---|
| 0.5 | 4.0 | 4.4 |
| " | 8.0 | 8.2 |
| " | 16.0 | 16.0 |
| " | 4.0 | 4.0 |
| " | 8.0 | 7.6 |
| " | 16.0 | 16.6 |
| " | 5.0 | 5.2 |
| " | 10.0 | 10.4 |
| " | 20.0 | 19.4 |
| 0.2 | 4.0 | 3.5 |
| " | 8.0 | 7.8 |
| " | 16.0 | 16.5 |
| " | 4.0 | 4.0 |
| " | 8.0 | 8.0 |
| " | 16.0 | 16.0 |
| " | 25.0 | 24.0 |

blood filtrate instead of 2 ml. and by the exclusion of water, giving an additional reduplication of color.

*Precipitation of Inulin.*—The amount of precipitant used was found to allow for quantities of inulin in plasma filtrates up to 100 micrograms. Attempts to dispense with the boiling for precipitation have yielded erratic results. Heat seems to be necessary to insure a stable relation between inulin and calcium hydroxide which will withstand subsequent washing.

*Blanks.*—The diphenylamine reagent develops a color with ageing and should be frequently prepared in small quantities. We found the urine blank values to be no higher than those of the reagent blank with water. A water blank may, therefore, be substituted for the urine blank proposed if no sugar or other abnormal constituents are present which produce color with the reagent. Serum blank values were equivalent to about 4 to 5 mg. per cent inulin. The same extinction was obtained by treating a solution of 100 mg. per cent glucose in water as described for plasma. It is thus mainly glucose which is responsible for the serum blank value. The proportion of 1:25 in color produced by glucose and inulin, respectively, would require a fluctuation in blood sugar of 25 mg. per cent in order to change the apparent inulin content by 1 mg. per cent. We consider ourselves, therefore, justified in dispensing with yeast treatment, except under very special experimental conditions.

*Recovery Experiments.*—Recovery experiments are presented in Table II.

*Application of the Method for Inulin Clearance Tests.*—With the described method for inulin determination, we found it entirely sufficient to inject only 1.5 or 2 Gm. intravenously instead of the usual 10 grams. This dose gives an initial serum level of 12 to 15 mg. per cent with sufficient time for several clearance periods before the concentration has fallen too low for determination. This should make inulin clearance studies more attractive to clinicians, who have been reluctant to undertake them because of the danger of pyrexial reactions. The higher color production makes clearance studies feasible even in small laboratory animals by reducing the quantity of plasma required for analysis.

SUMMARY

A method has been developed for the estimation of inulin in blood plasma and in urine. Inulin is precipitated with calcium hydroxide and the color elicited with the dry precipitate with diphenylamine. This increases greatly the color intensity obtained.

The method permits the reduction of the inulin dose injected for clearance tests in human beings and makes clearance tests feasible in small laboratory animals.

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# CHROMATOGRAPHIC SEPARATION OF CHOLESTEROL AND CHOLESTEROL ESTERS IN BLOOD

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THE methods commonly used for the determination of free cholesterol in blood depend upon its precipitation with digitonin followed by the development of color directly upon the digitonide, usually with the Liebermann-Burchard procedure.[1-3] The estimation of cholesterol esters, which are not precipitated by digitonin, should be done after saponification because, as has been repeatedly shown, the esters give more color in the Liebermann-Burchard method than does free cholesterol.[4-8] In the estimation of both free and ester cholesterol these two methods are combined.[2, 3] For complete precipitation of the cholesterol digitonide twenty-four hours are required, although Schoenheimer and Sperry[3] state that satisfactory results can be obtained with only one-hour standing. A method which would eliminate the costly and time-consuming digitonin precipitation is to be desired.

Trappe[9] used aluminum oxide to adsorb various lipids from petroleum ether solution and separated cholesterol stearate from free cholesterol by first eluting with trichlorethane to remove the ester and then with absolute ethyl alcohol to remove the cholesterol. He used large amounts of material, 71.2 mg. of cholesterol stearate and 72.2 mg. of cholesterol, and recovered 99.3 per cent of the stearate despite the fact that there was some saponification of the ester. A satisfactory method for blood analysis would require the separation and determination of much smaller quantities of cholesterol ester and free cholesterol. Subsequently Trappe[10, 11] described a method for the determination of both free and ester cholesterol in whole blood based, in part, upon these experiments. His procedure involved the use of both silica gel and aluminum oxide; however, no values for either plasma or serum were given.

Chromatographic procedures have been used in our laboratory for the separation of estrone from blood[12] and it seemed possible to devise a similar method for the determination of both free and ester cholesterol. This paper describes such a method based upon adsorption on aluminum oxide and upon selective elution followed by estimation by the Liebermann-Burchard method.

## METHOD

*Principle of the Method.*—Either plasma or serum is treated with a 1:1 aceteone-ethyl alcohol solution and filtered. An aliquot of the filtrate is evaporated to dryness dissolved in petroleum ether, and placed upon an aluminum oxide column. The ester fraction is eluted with a 10 per cent solution of ethyl ether in petroleum ether, and the free cholesterol is then eluted with a 10 per cent solution of ethyl alcohol in petroleum ether. The cholesterol esters are then saponified and the free cholesterol in both fractions is determined colorimetrically, using cholesterol as the standard.

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*Procedure.*—To approximately 20 ml. of a 1:1 acetone-ethyl alcohol solution in a 25 ml. volumetric flask add 1.0 ml. of plasma or serum. The suspension is brought to boiling on the water bath, cooled, and made to volume with the acetone-alcohol solution and filtered.[8] A 10 ml. aliquot of the filtrate is evaporated to dryness on the water bath and dissolved in 5.0 ml. of petroleum ether. The solution is placed upon the chromatographic column, containing 2.5 Gm. of aluminum oxide according to Brockman (Merck), and washed with 5.0 ml. of petroleum ether.[13] The ester fraction is eluted by the addition of 50 ml. of 10 per cent ethyl ether in petroleum ether. The eluate is collected and evaporated to dryness on a water bath. The free cholesterol is eluted with 25 ml. of 10 per cent ethyl alcohol in petroleum ether. This eluate is likewise evaporated to dryness.

The cholesterol ester fraction is dissolved in 10 ml. of 95 per cent ethyl alcohol and saponified by heating in an oven at 80 to 90° C. for ten minutes with 0.10 ml. of a potassium hydroxide solution (10 Gm. potassium hydroxide in 20 ml. water). This solution is neutralized with 10 per cent acetic acid in ethyl alcohol, using phenolphthalein as the indicator, one drop more than the required amount of acid is added. The solution is then evaporated to dryness on the water bath.

Both the free cholesterol and the saponified fraction are extracted with anhydrous chloroform and filtered into 10 ml. volumetric flasks. The filtrates are made to volume with chloroform. The reagent for color development is prepared by adding 1.0 ml. of concentrated sulfuric acid to 10 ml. of acetic anhydride. The acetic anhydride is chilled in an ice bath during the addition of the sulfuric acid and is kept there until used. The reagent is prepared fresh for each determination. To 5.0 ml. aliquots of the chloroform solutions is added 1.0 ml. of the reagent, and the color developed is read after standing for ten minutes. A Klett-Summerson colorimeter was used with either filter 54 or 42; the latter was found to be more sensitive.[14, 15] The color is stable for at least ten minutes.

## RESULTS

A standard solution containing 1.0 mg. of cholesterol in 5.0 ml. of petroleum ether was used to obtain a calibration curve. Aliquots of the standard solution were pipetted into colorimeter tubes, evaporated to dryness, and 5.0 ml. of chloroform added to each tube. Color development was carried out as described. The color obtained obeyed Beer's law in the concentration range of 0.02 to 0.50 mg. of cholesterol; above this concentration the color was too dense for accurate reading.

At the same time that the chromatographic separation was performed, another 10 ml. aliquot of the original plasma or serum filtrate was saponified and the total cholesterol determined by the method of Sperry and Brand.[8]

*Accuracy of Separation of Cholesterol From Its Ester.*—Cholesterol stearate was prepared by the method of Page and Rudy.[16] A petroleum ether solution of the ester and cholesterol was prepared containing 1.0 mg. of cholesterol stearate (equivalent to 0.60 mg. cholesterol) and 0.2 mg. of cholesterol in 5.0 milliliters. This ratio represents approximately that found in normal human serum. Twelve separate determinations were made upon aliquots of the solution using the procedure described. The recovery of free cholesterol was 0.21 ± 0.01 mg., and of ester cholesterol, 0.57 ± 0.025 milligram. This represents an accuracy of approximately 5 per cent. It might be noted, however, that the ester recovery generally tended to be about 5 per cent low and the free cholesterol recovery was high by about the same amount. In a series of experiments with free cholesterol alone, a similar plus or minus variation was observed whether

the cholesterol was chromatographed or run directly. This error is probably inherent in the colorimetric method.

*Recovery of Cholesterol Added to Blood Serum.*—Since it was possible to separate cholesterol from cholesterol stearate in pure solution, the separation was next attempted in blood serum. The same serum was analyzed before and after the addition of the ester and cholesterol to the alcohol-acetone solution of the serum extract before filtering. In a 25 ml. flask were placed 15 ml. of the acetone-alcohol solution and 1.0 ml. of petroleum ether containing 0.4 mg. of cholesterol and 1.7 mg. of cholesterol stearate (equivalent to 1.0 mg. of cholesterol), and then 1.0 ml. of serum was added. The procedure as detailed previously was employed. The results of these experiments are given in Table I. Cholesterol can be separated from cholesterol ester in blood serum with the same degree of precision as in pure solution.

TABLE I. RECOVERY OF CHOLESTEROL AND CHOLESTEROL STEARATE ADDED TO BLOOD SERUM

| SAMPLE | SERUM | | | | RECOVERY | |
|---|---|---|---|---|---|---|
| | FOUND | | FOUND AFTER ADDITION OF 0.40 MG. CHOLESTEROL AND 1.00 MG. CHOLESTEROL AS STEARATE | | | |
| | CHOL. (MG.) | ESTER (MG.) | CHOL. (MG.) | ESTER (MG.) | CHOL. (MG.) | ESTER (MG.) |
| 1 | 0.87 | 1.05 | 1.30 | 2.07 | 0.43 | 1.02 |
| 2 | 0.87 | 1.05 | 1.25 | 2.10 | 0.38 | 1.05 |
| 3 | 0.85 | 1.00 | 1.25 | 2.06 | 0.40 | 1.06 |
| 4 | 0.85 | 1.00 | 1.28 | 1.91 | 0.43 | 0.91 |
| Average | | | | | 0.41 | 1.01 |

In order to determine the reproducibility of the results, six separate determinations were made upon the same sample of blood serum. These results are given in Table II. The per cent of free cholesterol found varied from 33.3 to 36.6, with a standard deviation of 0.13 from the mean of 35.1 per cent. There is a deviation of 5 per cent from the mean for the highest and lowest values. The first column gives the data obtained upon the aliquot not chromatographed but analyzed after saponification by the method of Sperry and Brand.[8] In general the agreement between this method and the chromatographic method is good.

*Estimation of Cholesterol and Cholesterol Esters in Blood Serum and Plasma.*—In Table III are presented the data obtained upon ten samples of normal serum, and in Table IV, upon twenty-five samples of blood plasma. The average total amount of cholesterol present as determined by the Sperry-Brand[8] procedure is practically identical with the average total amount obtained by the chromatographic procedure, showing, at least, that there is no loss in the method. There is no significant difference in the ratio of free cholesterol to total cholesterol obtained in the plasma from that in the serum. The ratio is rather constant and is independent of the total amount of cholesterol present. As found by Schoenheimer and Sperry,[3] using digitonin as the precipitant, it is 31.3 expressed as per cent, based upon the data from seventeen subjects. In a subsequent paper Sperry[17] reported a value of 26.9 ± 1.4 per cent based upon data

TABLE II. REPRODUCIBILITY OF ESTIMATIONS OF FREE AND ESTER CHOLESTEROL IN THE SAME BLOOD SERUM

| TOTAL* (MG. %) | TOTAL (MG. %) | FREE (MG. %) | ESTER (MG. %) | $\frac{\text{FREE}}{\text{TOTAL}} \times 100$ (%) |
|---|---|---|---|---|
| 158 | 152 | 52 | 100 | 34.2 |
| 158 | 159 | 57 | 102 | 35.8 |
| 151 | 171 | 57 | 114 | 33.3 |
| 152 | 172 | 63 | 109 | 36.6 |
| 157 | 165 | 57 | 108 | 34.5 |
| 152 | 164 | 59 | 105 | 35.9 |
| Average 154.6 | 163.8 | 57.5 | 106.3 | 35.1 ± 0.13 |

*Method of Sperry and Brand.[8]

TABLE III. CHOLESTEROL AND CHOLESTEROL ESTERS IN NORMAL BLOOD SERUM

| TOTAL* (MG. %) | TOTAL (MG. %) | FREE (MG. %) | ESTER (MG. %) | $\frac{\text{FREE}}{\text{TOTAL}} \times 100$ (%) |
|---|---|---|---|---|
| 138 | 132 | 39 | 93 | 29.5 |
| 150 | 137 | 37 | 100 | 27.1 |
| 158 | 152 | 52 | 100 | 34.2 |
| 144 | 155 | 47 | 108 | 30.2 |
| 155 | 156 | 66 | 90 | 42.3 |
| 186 | 198 | 83 | 115 | 41.9 |
| 186 | 203 | 70 | 133 | 34.2 |
| 225 | 229 | 79 | 150 | 34.5 |
| 229 | 230 | 75 | 155 | 32.6 |
| 240 | 249 | 87 | 162 | 34.9 |
| Average 181.1 ± 37.6 | 184.1 ± 43.0 | 63 ± 18.0 | 120.6 ± 27.6 | 34.1 ± 4.9 |

*Method of Sperry and Brand.[8]

TABLE IV. CHOLESTEROL AND CHOLESTEROL ESTERS IN NORMAL BLOOD PLASMA

| TOTAL* (MG. %) | TOTAL (MG. %) | FREE (MG. %) | ESTER (MG. %) | $\frac{\text{FREE}}{\text{TOTAL}} \times 100$ (%) |
|---|---|---|---|---|
| 114 | 112 | 40 | 72 | 35.7 |
| 126 | 125 | 50 | 75 | 40.0 |
| 136 | 129 | 55 | 74 | 42.6 |
| 138 | 132 | 39 | 93 | 29.5 |
| 150 | 137 | 37 | 100 | 27.0 |
| 136 | 141 | 55 | 86 | 39.0 |
| 145 | 143 | 55 | 88 | 38.1 |
| 173 | 143 | 42 | 101 | 29.4 |
| 150 | 146 | 60 | 86 | 41.0 |
| 136 | 150 | 55 | 95 | 36.0 |
| 162 | 155 | 62 | 93 | 40.0 |
| 144 | 155 | 47 | 108 | 30.0 |
| 165 | 170 | 65 | 105 | 38.2 |
| 169 | 171 | 58 | 113 | 33.9 |
| 172 | 174 | 57 | 117 | 32.8 |
| 200 | 175 | 64 | 111 | 36.5 |
| 194 | 176 | 66 | 130 | 33.6 |
| 175 | 177 | 53 | 124 | 29.9 |
| 190 | 184 | 63 | 121 | 34.2 |
| 176 | 184 | 67 | 117 | 36.4 |
| 190 | 185 | 73 | 112 | 39.4 |
| 176 | 186 | 50 | 131 | 28.4 |
| 225 | 229 | 88 | 141 | 38.4 |
| 236 | 257 | 112 | 145 | 43.5 |
| 336 | 349 | 147 | 202 | 42.1 |
| Average 172.6 ± 45.5 | 172.6 ± 49.1 | 63.6 ± 24.3 | 109.0 ± 28.0 | 35.8 ± 4.7 |

*Method of Sperry and Brand.[8]

from sixty-two subjects, and he stated that with few exceptions values over 31 per cent were found only in the presence of infection or liver disease.

Using substantially the same method, Sobel and Mayer[18] found the percentage of free cholesterol to total cholesterol to vary from 22.6 to 29.2. On the other hand, Page and co-workers[19] found no such constancy of ratio, but their average value, based on data from sixty-six subjects, was 34.9 per cent free. Other workers[20] used digitonin to precipitate the free cholesterol, and the digitonide was estimated gasometrically. Folch and co-workers[21] have stated that the gasometric values for free cholesterol are 20 per cent too high. Gardner and Gainsborough[22] likewise used digitonin to precipitate free cholesterol which was then estimated colorimetrically and reported 35.3 per cent free cholesterol in twenty-one women and 33.8 per cent in twenty-two men. Recently Foldes and Murphy,[23] using digitonin precipitation, found an average of 33 per cent free cholesterol in the blood plasma of forty normal subjects. Although there appears to be some difference in the percentage of free cholesterol obtained by different laboratories using various methods, it is apparent that there is little disagreement over the fact that the percentage of free cholesterol is quite constant. The chromatographic method gives values for the percentage of free cholesterol within the range of those reported by others.

## SUMMARY

Cholesterol and cholesterol esters adsorbed upon aluminum oxide from petroleum ether solution can be separated by selective elution. The ester fraction is eluted with 10 per cent ethyl ether dissolved in petroleum ether, and the free cholesterol with a 10 per cent solution of ethyl alcohol in petroleum ether.

The ester fraction is saponified and the free cholesterol in both fractions is determined by the Liebermann-Burchard colorimetric method. The method is rapid, approximately three hours are required for a complete determination, and the results are reproducible with an accuracy within 5 per cent.

The procedure has been applied to normal blood serum and plasma. The per cent of free cholesterol found in ten normal sera averaged 34.1 ± 4.9 per cent, and in twenty-five normal plasmas it averaged 35.8 ± 4.7 per cent of the total cholesterol.

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